Investigations of Multiply-Resistant Enterococci in Hong Kong

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Abstract

Enterococci have recently emerged as important pathogens because of their association with nosocomial infections and their antimicrobial multiple resistance traits. Since the situation of enterococcal infections and their resistance in Hong Kong is largely unknown, we undertook a study to determine the species distribution and antimicrobial susceptibilities of clinical enterococcal isolates in the Prince of Wales Hospital from 1997 to 1998.

A total of 498 non-replicate enterococcal isolates were studied. These included 397 isolates and 44 isolates from urine and bile, respectively, collected during the three-month period (April – June) of 1997 and 1998; and 49 isolates from blood, two isolates from cerebrospinal fluid and six isolates from body fluids from April 1997 to March 1998. Our finding that the majority of enterococcal isolates were from urine was similar to that reported in other studies. The ability of polymerase chain reaction (PCR) to definitively identify enterococcal isolates to species level was evaluated by testing 309 isolates (which represented more than 60% of the total isolates studied and were thus a representative collection of enterococcal strains) using API 20 Strep, PCR and conventional biochemical tests. These isolates were from urine and bile collected during April - June, 1997 and from blood, body fluid and cerebrospinal fluid collected during April, 1997 - March, 1998.

Both API 20 Strep and PCR gave the same identification results for 240 *Enterococcus faecalis* isolates, 45 *E. faecium* isolates and one isolate each of *E.*

gallinarum and *E. casseliflavus*. The remaining 69 isolates were re-identified by conventional biochemical tests. It was found that PCR could correctly identify 303 isolates while API 20 Strep could only correctly identify 287 (93%) isolates, i.e., 99% of *E. faecalis* and 57-87% of the other *Enterococcus* species. Thus, PCR was used in the identification of the remaining isolates and the identity of isolates other than *E. faecalis* was subsequently confirmed by conventional biochemical tests. Results indicated that 403 (81%) were *E. faecalis*, 77 (15%) were *E. faecium* and 18 (4%) were other enterococcal species (*E. gallinarum* (8), *E. casseliflavus* (4), *E.durans* (2), *E. raffinosus* (3) and *E. avium* (1)). This distribution of clinical enterococcal species was similar to that found in other parts of the world.

E. faecium isolates were more frequently resistant than *E. faecalis* to penicillin (95% versus 1%), ampicillin (87% versus 1%), high-level streptomycin (67% versus 36%), trimethoprim (53% versus 16%), co-trimoxazole (58% versus 15%), rifampicin (92% versus 56%) and sparfloxacin (66% versus 8%). The percentage of other enterococcal species resistant to these antibiotics was in general similar to that of *E. faecalis*. Only four isolates were resistant to vancomycin, two were *E. faecium* (vancomycin MIC >16 mg/L) and one each of *E. gallinarum* and *E. casseliflavus* (vancomycin MIC = 8 mg/L). One of the vancomycin-resistant *E. faecium* was also resistant to teicoplanin (MIC >16mg/L). Thus, vancomycin resistance was not a problem here as compared to the United States and some European countries where VRE were prevalent. Our study showed that an average of 15% of the enterococcal isolates were resistant to ampicillin and 25% were resistant to high concentrations of gentamicin. If the standard regimen of

ampicillin plus an aminoglycoside for treating serious enterococcal infections was used, there would be a 1 in 4 chance of treatment failure. In general, resistance to antimicrobials other than vancomycin and teicoplanin of enterococci in Hong Kong was high as compared to that in other countries.

A total of 47 isolates with vancomycin MICs ≥4 mg/L were tested for the presence of vancomycin resistance genes by PCR. One isolate each of *E. faecium* was shown to harbour *vanA* and *vanB* genes. The *vanC-1* gene was found in eight *E. gallinarum* isolates and *vanC-2* gene was found in two *E. casseliflavus* isolates. One hundred and twenty-four isolates with gentamicin MICs ≥1024 mg/L were subjected to PCR to detect for the presence of the *aac(6')-aph(2")* gene. Only four isolates did not harbor the gene. None of the ampicillin-resistant enterococci (ampicillin MIC >8mg/L) produced β-lactamase. Pulsed-field gel electrophoresis (PFGE) of *Sma*I-restricted DNA of multi-resistant enterococci showed that they were heterogeneous and no obvious outbreak had occurred in the hospital.

內容撮要

近年由於醫院裏的病人經常感染腸球菌，以及它對常用的抗生素呈耐藥性，腸球菌漸成爲一種常見於醫院感染病的細菌。由於本港缺乏對腸球菌的研究，所以希望藉此研究了解本港的腸球菌分佈及其耐藥情況。

本項研究共收集了四百九十八株腸球菌，這些菌株是在一九九七年及一九九八年的其中三個月內(四至六月)所收集得。其中三百九十七株是從尿液中分離及四十四株從膽汁中分離。另外，從一九九七年四月至一九九八年三月的一年中，共收集了五十七株菌種，分別有四十九株是從血液，有六株是從各種體液及有兩株從腦脊液中分離。

爲了測試聚合霉鏈反應(PCR)能否準確地監定腸球菌，我們將其中之三百零九株菌種，用API 20Strep、聚合霉鏈反應及發霉測試等方法來作鑒別。API 20Strep及聚合霉鏈反應均能準確地鑒別出其中之二百四十株爲*E. faecalis*，四十五株爲 *E. faecium* 及*E. gallinarum* 和 *E. casseliflavus* 各一株，餘下之菌種由於被API 20 Strep和聚合霉鏈反應分別鑒定成不同種，所以需要以發酵測試重新鑒別這些菌種。結果顯示，聚合霉鏈反應能準確地鑒別了三百零三株腸球菌，而API 20 Strep只能準確鑒別出二百八十七株 (93%)分。 API 20 Strep 能準確地鑒定出99%的*E. faecalis*，但用來鑒定其它的腸球菌則只有57 – 87%的準確性。因此我們選用聚合霉鏈反應來鑒定餘下的菌種，若菌株被鑒定爲非*E. faecalis*時，我們會利用發酵測試再作鑒定。我們所收集的四百九十八株菌種，有四百零三株 (81%) 是*E. faecalis*、七十七株 (15%) 是*E. faecium*、八株是*E. gallinarum*、四株是*E. casseliflavus*、兩株是*E. durans*、三株是*E. raffinosus*及一株是*E. avium*。本研究所得腸球菌的分佈與其他國家的

發現大致一樣。

另外，本研究又測試了腸球菌對不同抗生藥的耐藥性。*E. faecium*普遍比*E. faecalis*對以下抗生素耐藥，青霉素 (95% 對1%)，氨苄西林 (87% 對1%)，鏈霉素 (67% 對36%)，甲氧苄氨嘧啶 (53% 對16%)，復方新諾明 (58% 對15%)，利福平 (92% 對56%) 及sparfloxacin (66% 對8%)。其它腸球菌的耐藥程度跟*E. faecalis* 的大致相若。只有四株菌種對萬古霉素呈耐藥，其中兩株爲 *E. faecium* (MIC >16 mg/L) 及 *E. gallinarum, E. casseliflavus* 各一株 (MIC = 8 mg/L)。而其中一株對 萬古霉素呈耐藥的 *E. faecium* 對 teicoplanin 同樣呈耐藥 (MIC >16mg/L)。

在對萬古霉素(MIC ≥4 mg/L) 呈耐藥的四十七株腸球菌裏，發現只有兩株*E. faecium*含有*vanA*及*vanB*的基因。另外，有八株*E. gallinarium*含有*vanC1*的基因及兩株*E. casseliflaus*含有*vanC2*的基因。這些*van*基因分別利用特定引物在聚合霉鏈反應中測試出來。我們也利用聚合霉鏈反應測試了一百二十四株對慶大霉素呈高度耐藥 (MIC ≥1024 mg/L) 的菌種。其中只有四株沒有存在對慶大霉素呈耐藥的基因 (*aac6'-aph2*)。而在對氨苄西林 (MIC >8 mg/L) 耐藥的菌種中，並沒有一只製造 β-內酰胺酶 (β-lactamase)。另外，又用核酸內切酶 (*Sma*I) 消化屬於對多種抗生素呈耐藥的腸球菌的脫氧核糖核酸，經pulsed-field gel electrophoresis (PFGE)，後再作圖譜分析。發現可能有多個腸球菌的克隆存在本醫院中，但並沒有顯示有暴發感染。

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1. Introduction

1.1. Taxonomy of enterococci

The term "enterococcus" was first used by Thiercelin in 1899 to describe those bacteria seen in pairs or short chains in faeces (Murray, 1990). With the development of Lancefield's streptococcal grouping system and the discovery of group D antigen in enterococci in the 1930s, the concept of enterococci as salt tolerant group D streptococci was established (Ruoff *et al*, 1990). In 1937, Sherman divided the serological group D streptococci into two groups: I) 'enterococcus' that included *Streptococcus faecalis* and *S. faecium*, II) 'non-enterococcus' that included *S. bovis* and *S. equinus* (Murray, 1990).

Recently, the development and widespread use of molecular techniques in taxonomic analysis have led to a significant change in the classification of enterococci. In 1970, Kalina suggested to put *S. faecalis* and *S. faecium* and their subspecies into a separate genus - *Enterococcus* (Kalina, 1970). His suggestion was based on their cellular arrangement and phenotypic characteristics. However, the genus *Entercoccus* was not established until 1987 when Schleifer and Kilpper-Balz noted that *S. faecalis* and *S. faecium* were not related to streptococci by DNA-DNA and DNA-rRNA hybridization (Schleifer & Kilpper-Balz, 1984 & 1987). There were initially only two species in this genus - *E. faecalis* and *E. faecium* but now, eleven more species are described. These include *E. durans, E. avium, E. casseliflavus, E. malodoratus, E. gallinarum, E. mundtii, E. raffinosus, E. solitarius, E. hirae, E. pseudoavium and E. flavascens*.

1.2. Natural habitat of enterococci

Enterococci are found naturally in the environment such as soil and water and in animals and insects. In humans and in animals, enterococci are present as normal flora in the gastrointestinal and genital tracts. *E. faecalis* is amongst the most common bacteria found in the human gut while *E. faecium* may also be commonly found (Facklam & Teixeira, 1999).

1.3. General characteristics of the genus *Enterococcus*

Enterococci are gram-positive, facultative anaerobic cocci that occur singly or are arranged in pairs or short chains. The optimum growth temperature is 35°C but most of them can grow at a temperature range of 10°C and 45°C. They are similar to streptococci in that they do not have cytochrome enzymes and so are catalase negative. All enterococci can grow in 6.5% sodium chloride broth and hydrolyse esculin in the presence of 40% bile salts. In addition, they can hydrolyse L-pyrrolidonyl-β-naphthylamide (PYR) and produce leucine aminopeptidase (LAP).

1.4. Identification to species level of enterococci

The identification of enterococci to species level is often required for epidemiological investigations. Enterococci are conventionally speciated using biochemical and physiological tests. According to Facklam and Collins (1989), enterococci can be differentiated into three groups based on acid formation in mannitol, sorbitol, and sorbose broth and hydrolysis of arginine.

E. avium, E. malodoratus, E. raffinosus and *E. pseudoavium* belong to group I. They form acid in all the above three carbohydrate broths but fail to hydrolyze arginine. They can be further differentiated by acid formation in arabinose and raffinose broths. *E. avium* forms acid in arabinose but not in raffinose broth. *E. malodoraus* forms acid in raffinose but not in arabinose broth. *E. raffinosus* forms acid in both while *E. pseudoavium* does not form acid in either broths.

Group II includes *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. mundtii*, *E. flavescens* and *E. gallinarum*. They form acid in mannitol broth and hydrolyze arginine but fail to form acid in sorbose broth and give variable reactions in sorbitol broth. Tolerance to tellurite, utilization of pyruvate, motility test and yellow pigment formation can be used to further differentiate them. *E. faecalis* is the only species in this group that tolerates tellurite and utilizes pyruvate. *E. faecium* and *E. gallinarum* have similar characteristics but they can be differentiated by motility test : *E. faecium* is non-motile but *E. gallinarum* is motile. *E. casseliflavus*, *E. mundtii* and *E. flavescens* produce yellow pigment. However, *E. casseliflavus* and *E. flavescens* are motile while *E. mundtii* is not. *E. casseliflavus* forms acid in ribose broth while *E. flavescens* does not.

Group III includes *E. durans*, *E. hirae* and *E. dispar*. They can hydrolyze arginine but do not form acid in mannitol, sorbitol or sorbose broth. They can be further differentiated by the pyruvate, raffinose and sucrose tests. *E. durans* is

negative in all three tests. *E. hirae* can form acid in one or both broths but does not utilize pyruvate. *E. dispar* is positive in all three tests.

Identification of enterococci by conventional biochemical tests is often tedious and labour intensive. Commercial identification systems are much simpler to use and include API 20 Strep system, Gram Positive Identification Card of Vitex System, API 32 rapid Strep system (all from bioMerieux SA, Montalieu Vercieu, France) and RapID STR system (Innovative Diagnostic Systems, Atlanta, Ga., USA). Most of these systems provide results in approximately four hours. These systems had been evaluated and were shown to be able to accurately identify *E. faecalis* but not the other enterococcal species (Kuoff & Kung, 1982; Ruoff *et al*, 1982; Ruoff, 1990; Nachamkin, Lynch & Dalton, 1982; Jorgensen Crawford & Alexander, 1983; Appelbaum *et al,* 1984a, 1984b & 1986; Facklam, Rhoden & Smith, 1984; Facklam *et al*, 1985; You & Facklam, 1986; Fertally & Facklam, 1987).

Accurate identification based on biochemical tests might be difficult to achieve for some species with unusual biochemical characteristics or for those that differ by only one or two biochemical properties (Williamson *et al*, 1986; Facklam & Collins, 1989; Ruoff *et al*, 1990; Vincent *et al*, 1991; Donabedian *et al*, 1995(a); Facklam, Sahm & Teixeria, 1999). Examples include *E. faecium* with unusual sorbitol and sorbose fermentation characteristics (Williamson *et al*, 1986), *E. gallinarum* and *E. casseliflavus* are motile while *E. faecium* and *E. faecalis* are not,

and *E. casseliflavus* usually forms yellow pigment while *E. gallinarum* does not (Facklam & Collins, 1989). Besides, there are some strains of *E. casseliflavus* that are motile and do not produce pigment (Vincent *et al*, 1991). Recently, molecular biological techniques had been used to identify enterococci. These included nucleic acid hybridization, DNA amplification and protein analysis (penicillin binding proteins and whole cell profiles).

Polymerase chain reaction (PCR) is one of the most common techniques used. Primers for the specific identification of *E. faecalis* and *E. faecium* have been designed (Dutka-Malen, Evers & Courvalin, 1995). Since the *vanC-1* and *vanC-2/C-3* genes are found only in *E. gallinarum* and *E. casseliflavus/E. flavescens,* respectively, primers specific for *vanC-1* and *vanC-2/3* genes are used for the identification of these two species (Leclercq *et al*, 1992; Dutka-Malen, Evers & Courvalin, 1995; Clark *et al*, 1998). The method was based on the specific detection of genes coding for D-alanine:D-alanine (D-Ala:D-Ala) ligases (ddl) present in all *E. faecalis* and *E. faecium* ($ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$, respectively) but not in other enterococcal species (Dutka-Malen, Evers & Courvalin, 1995). The D-ala-D-serine ligases are encoded by the intrinsic genes *vanC-1* and *vanC-2* or *vanC-3* found in *E. gallinarum and E. casseliflavus* or *E. flavescens,* respectively but not in other enterococcal species tested (Leclercq *et al*, 1992; Navarro & Courvalin, 1994, Cartwright *et al*, 1995). The *van C-2* and *van C-3* genes of *E. casseliflavus* and *E. flavescens* are almost identical (98%) and primers for *van C-2* can also anneal to *van C-3* (Navarro & Courvalin, 1994; Clark *et al*, 1998). Thus, PCR

cannot be used to distinguish these two species which are phenotypically very similar (Dutka-Malen, Evers & Courvalin, 1995; Patel *et al*, 1997).

1.5. Clinical significance of enterococci

Enterococci are normal flora of the gut, vagina, perineum, anterior urethra and oral cavity (Nord & Wadström, 1973; Adhami, 1982; Facklam, Sahm & Teixeria, 1999). They are regarded as being of low pathogenicity because they lack the common virulence factors such as haemolysin, aggregation substances and adhesives (Facklam & Teixeria, 1999; Witte, Wirth & Klare, 1999). However, these organisms have increased in importance as nosocomial pathogens during the past 20 years in many parts of the world. They become the second most common nosocomial pathogens in the United States, accounting for 10% of all hospital-acquired infections in 1986-1989 (Schaberg, Culver & Gaynes, 1991). *E. faecalis* is the most common enterococcal species causing infections in man, followed by *E. faecium* (Silverman *et al*, 1998; Reinhert *et al*, 1999; Schouten *et al*, 1999). The other species do not frequently cause infections. From a recent report by the National Nosocomial Infections Surveillance (NNIS) system, enterococci were reported as the second most common pathogens isolated from bloodstream infection and the third most common pathogens isolated from urinary tract infection in the intensive care unit (ICU) in the United States during 1990-1999 (CDC, 1999).

Most of the enterococcal infections are endogenous, that is, they are acquired from the patient's own flora, and most of them are naturally resistant to multiple antibiotics. Thus, extensive use of broad-spectrum antimicrobial agents such as cephalosporins and quinolones that had no activity against enterococci was thought to predispose patients to the development of enterococcal infections (Spera & Farber, 1992; Heath, Blackmore & Gordon, 1996; Hunt, 1998). However, the clinical importance of enterococci has often been questioned except when they are isolated from urine, blood or other sterile sites (Murray, 1990; Maki & Agger, 1988; Low *et al*, 1994).

Enterococci can cause various diseases, the most common being urinary tract infections, followed by intra-abdominal or pelvic wound infections, bacteraemia, endocarditis and neonatal meningitis. Enterococci account for about 10% of all urinary tract infections (Felmingham *et al*, 1992). They are second to *Escherichia coli* as causes of nosocomial urinary tract infections, urinary catheterization or instrumentation being the important predisposing factors (Murray, 1990; Schaberg, Culver & Gaynes, 1991; Moellering, 1992).

Intra-abdominal or pelvic wound infections are the next most common infections caused by enterococci. A mixture of organisms usually causes these infections and the clinical significance of enterococci thus remains controversial (Dougherty, 1984; Nichols & Muzik, 1992). Use of a single antimicrobial agent

either for prophylaxis or treatment of these infections is probably an important predisposing factor (Low *et al*, 1994; Low, Willey & McGeer, 1995).

Enterococci are the third most common cause of nosocomial bacteraemia in the United States in 1986-1989 (Schaberg, Culver & Gaynes, 1991). More than 40% of such bacteraemia are polymicrobial, caused by *Enterococcus* and one other microorganism and the source being mainly from the urinary tract, intra-abdominal or pelvic sepsis, wound or intravenous catheter (Maki & Agger, 1988; Gullberg, Homann & Phair, 1989). Patients are usually elderly and have serious underlying diseases or immunocompromised and have prolonged hospital stay and on antimicrobial therapy. Mortality of patients with enterococcal bacteremia before VRE appeared was 33%-68% (Edmond *et al*, 1996(b)). However, it is interesting to note that mortality rate attributable to bacteremia caused by vancomycin-susceptible enterococci (VSE) and VRE is similar, being 31% and 37%, respectively (Landry, Kaiser & Wenzel, 1989; Edmond *et al*, 1996(a)). It could be postulated that strains of VRE were probably not inherently more virulent than VSE.

In contrast to enterococcal bacteremia, which is frequently polymicrobial, enterococcal endocarditis is usually not. However, it is significantly associated with underlying valvular heart disease. About 5% to 15% of all cases of endocarditis are caused by enterococci, the most common species being *E. faecalis* (Maki & Agger, 1988; Murray, 1990; Johnson *et al*, 1998). Enterococcal endocarditis is often sub-

acute and usually occurs in elderly men, young women and intravenous drug users with underlying valvular heart disease or prosthetic valves (Maki & Agger, 1988; Murray, 1990; Moellering, 1997).

1.6. Clinical management of enterococcal infections

Single-drug therapy such as penicillin, ampicillin or vancomycin is used for the treatment of uncomplicated enterococcal infections such as urinary tract infection (Anonymous, 1992). However, for serious enterococcal infections that require a bactericidal effect such as endocarditis, meningitis or osteomyelitis, treatment includes a cell wall active agent (such as penicillin, ampicillin or a glycopeptide) combined with an aminoglycoside (usually gentamicin or sometimes streptomycin) (Geraci & Martin, 1954; Koenig & Kaye, 1961; Moellering, 1992; Murray, 1990; Hunt, 1998).

This combination of therapy can overcome the intrinsic resistance to aminoglycoside of enterococci since penicillin disrupts the cell wall and increases the permeability of the organism to aminoglycoside thus resulting in an effective synergistic killing. However, strains have become resistant to high-levels of aminoglycosides so that such synergistic effect will be lost. With the development of resistance to an increasing number of antimicrobial agents, the choice of drugs for serious enterococcal infections has become limited (Spera & Farber, 1992; Heath, Blackmore & Gordon, 1996; Huycke, Sahm & Gilmore, 1998).

1.7. Antimicrobial susceptibilities of enterococci

Enterococci are relatively resistant to antimicrobial agents that are usually used in the treatment of gram-positive infections. They are also intrinsically resistant to many antimicrobial agents including the β-lactams and aminoglycosides. The minimum inhibitory concentrations (MICs) of β-lactam drugs are relatively high as compared to those to streptococci, e.g. the MIC of penicillin for enterococci is 10- to 100-fold higher than that for streptococci (Gordon *et al*, 1992; Low *et al*, 1994).

In general, *E. faecium* is more resistant to antimicrobial agents than *E. faecalis*. *E. faecium* is less sensitive to β-lactams than *E. faecalis*. However, *E. faecalis* is tolerant to penicillin or ampicillin and will not be killed unless concentrations reach 100 mg/L or higher. Acquired ampicillin-resistance in *E. faecalis* is rare and the first ampicillin-resistant *E. faecalis* strain due to the production of β-lactamase was not isolated until early 1980s (Murray, & Mederski-Samoraj, 1983; Gray & Pedler, 1992). Subsequently, high-level β-lactam-resistant enterococci dramatically increased and by the late 1980s, 8-78% of enterococci were ampicillin-resistant (Oster *et al*, 1990; Grayson, *et al*, 1991; Boyce *et al*, 1992; Patterson *et al*, 1995; Landman & Quale, 1997). Recent studies showed that the percentage of ampicillin-resistance in *E. faecalis* is 0-15% and in *E. faecium* about 5-80% (Gray, Stewart & Pedler, 1991; McNamara, King & Smyth, 1995; Vandamme *et al*, 1996; Iwen *et al*, 1997). The percentage of antibiotic resistance in enterococci also varies among countries. In New Zealand, all *E. faecalis* strains are susceptible to ampicillin but 10% of *E. faecium* are resistant (Taylor *et al*, 1997).

However, in the United States and European countries, 20-60% of *E. faecium* are resistant to ampicillin (Douboyas & Tsakris, 1995; Perlada, Smulian & Cushion, 1997; Reinert *et al*, 1999).

Vancomycin-resistant enterococci (VRE) were first seen in the late 1980s in Europe, the United States and Australia (Anderson, Davey & Bell, 1997; Moellering, 1997; Bell, Paton & Turnidge, 1998; French, 1998; Hunt, 1998;). Subsequently, more countries reported the presence of VRE. In general, more E. *faecium* strains are resistant to glycopeptides than is *E. faecalis*. Besides, many VRE strains are also resistant to all clinically important antibiotics (CDC, 1993). In Asian countries, the percentage of VRE remains low. In Japan, no *vanA* or *vanB* genes are detected and less than 1% of low-level vancomycin-resistant enterococci (van C type) are seen (Hirkata *et al*, 1997). Recently, high-level vancomycin-resistant enterococci were isolated in Taiwan (Hsueh *et al*, 1999). Although VRE are present in the United Kingdom and France, the incidence rate remains low (Leclercq, 1996; Johnson *et al*, 1998; Reinert *et al*, 1999). However, high incidence of VRE was reported in the United States. From the data of NNIS, the incidence of VRE in hospitals has increased dramatically from 0.3% in 1989 to 8% in 1993 (CDC, 1993).

High-level aminoglycoside resistance (HLAR) was first described in the 1970s in France (Hunt, 1998) and by mid-1980s, reports of HLAR in enterococci appeared all over the world with incidence varying from 10% to 60% (Gordon *et*

al, 1992; Douboyas & Tsakris, 1995; Vandamme *et al*, 1996; Tsakris, Pournaras & Douboyas, 1997; Johnson *et al*, 1998; Cirak & Sultan, 1998; Reinert *et al*, 1999; Papaparaskevas *et al*, 2000). In Asia, the percentage of HLAR varies, being 14% in Thailand, 22% in Singapore, 43% in Japan and 63% in Taiwan (Tsaur *et al*, 1993: Chiew & Hall, 1998). In European countries, the percentage of HLAR is 10% to 50% while in the United States, it is 10-60% (Gordon *et al*, 1992; Perlada, Smulian & Cushion, 1997; Tsakris, Pournaras & Douboyas, 1997; Johnson *et al*, 1998; Reinert *et al*, 1999). In some other countries like Germany, Australia and Belgium, the percentage of HLAR is similar in *E. faecium* and *E. faecalis* whereas in the United States, United Kingdom and Ireland, the percentage of HLGR in *E. faecium* is higher than *E. faecalis* (McNamara, King, Smyth, 1995; Vandamme *et al*, 1996; Iwen *et al*, 1997; Perlada, Smulian & Cushion, 1997; Reinert *et al*, 1999).

1.8. Antimicrobial resistance of enterococci

Antimicrobial resistance in enterococci can be either intrinsic or acquired. Enterococci are intrinsically resistant to different classes of antimcrobials including β-lactams, clindamycin, the folate inhibitors and aminoglycosides. Resistance to β-lactams is generally low-level and is mainly due to the low affinity of cell wall penicillin-binding proteins (PBPs) to these drugs (Williamson *et al*, 1983; Zighelboim-Duam & Moellering, 1988; Gray & Pedler, 1992). Although some β-lactams are active, they are not bactericidal against most of the strains. Furthermore, cephalosporins are ineffective against the enterococci (Leclercq, 1997).

Low-level aminoglycoside resistance is due to reduced uptake of drug by bacteria. Since aminoglycosides are highly positively charged and insoluble in organic solvents, they are unable to transverse the cytoplasmic membrane of enterococci. Besides, the anaerobic metabolism of enterococci does not favour transport of aminoglycosides which requires oxidative energy (Bryan, Kowand & van den Elzen, 1979). Despite their resistance to β-lactams and aminoglycosides, synergistic activity of the combination of these two classes of drugs has been demonstrated in the treatment of invasive enterococcal infections (Geraci & Martin, 1954; Moellering & Weinberg, 1971). Enterococci are shown to be susceptible to trimethoprim and co-trimoxazole in vitro. However, they may utilize exogenous supplies of thymidine and thymine and become resistant (Murray, 1990; Gray & Pedler, 1992).

In addition to their inherent antibiotic resistance, enterococci can very readily acquire resistance to a wide range of antimicrobial agents. Acquired resistance occurs either through mutation in the existing genes or through acquisition of new genes borne on plasmids or transposons (Murray, 1990). High-level resistance to β-lactams can be due to acquisition of a β-lactamase gene originating from staphylococci but most commonly due to an increase in the production of low-affinity penicillin-binding proteins (Murray, 1992; Fontena *et al*, 1992). β-Lactamase-producing *E. faecalis* was first isolated in the early 1980s in the United States and later in Argentina and Lebanon (Moellering, 1992; Murray, 1992). This type of resistance is usually plasmid-mediated and often linked with

high-level gentamicin resistance (Murray, 1992). *E. faecium* is less sensitive to ampicillin than *E. faecalis* due to differences in the PBPs. The increase in high-level β-lactam resistance is mainly due to overproduction of a penicillin-binding protein, PBP5, that has a low affinity for β-lactam drugs (Williamson *et al*, 1985; Herman & Gerding, 1991; Low, Willey & McGeer, 1995; Leclercq, 1997; Rybkine *et al*, 1998).

High-level resistance to the aminoglycosides in enterococci was first reported in the 1970s in France (Horodniceanu *et al*, 1979) and subsequently in many parts of the world (Murray, 1990; Patterson & Zervos, 1990; Low *et al*, 1994; Chiew & Hall, 1998). The spread of this resistance is usually due to the transfer of genes coding for aminoglycoside-modifying enzymes (AMEs). These genes are borne on plasmids or on transposons, some of which may be identical to those found in staphylococci (Patterson *et al*, 1988; Herman & Gerding, 1991; Hodel-Christian & Murray, 1991; Leclercq *et al*, 1992; Thal *et al*, 1994). However, in some strains of *E. faecalis*, high-level streptomycin resistance can also be due to gene mutation leading to a change in the affinity of ribosomes to streptomycin (Eliopoulous *et al*, 1984). The most common AME produced by enterococci is 6'-acetyltransferase 2" phosphotransferase (AAC(6')-APH(2")), a bifunctional enzyme. AAC(6')-APH(2") is the product of two AME genes, *aac-6'* and *aph-2"* which have fused to generate a single gene. It confers resistance to all clinically important aminoglycosides except streptomycin (Courvalin, Carlier & Collatz, 1980; Leclercq, 1997). Other AMEs produced by enterococci include streptomycin

adenylytransferase (ANT(6)), 3'-phosphotransferase (APH(3')) and 4'-nucleotidytranferase (ANT(4')). Strains that are resistant to high-levels of aminoglycosides will also be resistant to the synergistic activity of aminoglycosides plus cell wall active agents. Recently, another aminoglycoside resistance gene, *aph(2")-Ic*, coding for APH(2")-Ic enzyme which confers moderate level of resistance to gentamicin (MIC = 256 mg/L) has been detected in enterococci (Chow *et al*, 1997; Tsai *et al*, 1998).

Resistance to glycopeptides is due to various mechanisms and is classified as class A to E, based on the level of resistance to vancomycin and teicoplanin and whether the resistance is inducible or constitutive (Leclercq, 1988; Murray, 1990; Leclercq *et al*, 1992; Dutka-Malen, Evers & Courvalin, 1995; Free & Sahm, 1996; Leclercq, 1997; Moellering, 1998). Glycopeptides inhibit cell wall synthesis by binding to the terminal dipeptide, D-alanyl-D-alanine (D-ala-Da-ala) of peptidoglycan precursors at the cell surface which block this precursor from further participating in cell wall synthesis. Resistant strains have modified terminal dipeptide, which does not bind to glycopeptides.

Class A strains have inducible resistance to high-levels of both vancomycin (MIC ≥64 mg/L) and teicoplanin (MIC ≥16 mg/L) due to the presence of a *vanA* gene (Low, Willey & McGeer, 1995; Murray, 1997; Hunt, 1998). This gene is present on a plasmid and can be transferred to other Gram-positive bacteria (Gray & Pedler, 1992; Murray, 1997; French, 1998). It encodes a D-alanine-D-alanine

ligase of modified specificity that results in the synthesis of cell wall precursors ending in D-alanyl-D-lactate (D-ala-D-lac) instead of D-ala-D-ala, the target site for vancomycin (Arthur & Courvalin, 1993; Arthur, Reynolds & Courvalin, 1996; Murray, 1997).

Class B strains show moderate resistance to vancomycin (MIC 4 – 1024 mg/L) but remain susceptible to teicoplanin (MIC ≤1 mg/L) and have the *vanB* gene. This resistance phenotype is induced by vancomycin only and is transferable by conjugation in certain strains (Quintiliani, Evers & Courvalin, 1993; Arthur, Reynolds & Courvalin, 1996; Murray, 1997; Leclercq, 1997).

Class C strains show constitutive low-level resistance to vancomycin (MIC ≥8 to 32 mg/L) but are susceptible to teicoplanin. The resistance is a special characteristic of *E. gallinarum* (*vanC-1* genotype), *E. casseliflavus* (*vanC-2* genotype) and *E. flavescens* (*vanC-3* genotype). Since transferability of *vanC* gene has not been demonstrated, it is thought that the gene might be borne on the chromosome (Leclercq *et al*, 1992; Navarro & Courvalin, 1994; Murray, 1997; Toye *et al*, 1997; Clark *et al*, 1998).

Two new vancomycin resistance phenotypes have been discovered recently. *VanD* gene was reported in 1997 in an *E. faecium* isolate constitutively resistant to vancomycin and to low-level teicoplanin (Perichon, Reynolds & Courvalin, 1997). *VanE* gene was detected in an *E. faecalis* isolate that was resistant to low-levels of

vancomycin but not to teicoplanin (Fines *et al*, 1999). The sequence of the gene is 55% identical to *van C-1* gene (Fines *et al*, 1999).

Enterococci can also acquire resistance to other antibiotics such as the macrolides, choramphenicol, tetracycline and quinolones. Resistance to macrolides is due to the presence of a gene of coding for rRNA methylase that methylates 23S rRNA resulting in reduced ribosomal binding and subsequent resistance to lincoamide and streptogramin B (Murray, 1990; French, 1998). Chloramphenicol resistance in enterococci is usually due to the production of chloramphenicol acetyltransferase and is plasmid-mediated (Murray, 1990). Strains that are resistant to tetracycline harbour genes such as *tetM* and *tetN* that protect the ribosomes from inhibition by tetracycline (Leclerq, 1997). Quinolone resistance in enterococci is due to chromosomal mutation that alters the subunit A of DNA gyrase (French, 1998).

1.9. Molecular typing of enterococci

Traditional methods for the typing of enterococci include biotyping, antibiotic resistance profiling, serotyping, phage typing and bacteriocin typing. Biotyping, antibiotic resistance profiling and serotyping are shown to be insufficiently discriminatory for most epidemiological studies (Murray, 1990). Although phage typing and bacteriocin typing are superior, the procedures involved are laborious and special reagents and continuous maintenance are required (Murray, 1990).

Recently, various molecular techniques such as plasmid profile analysis, ribotyping and DNA fingerprinting have been used in the typing of enterococci. Plasmid analysis, whereby the number and size of plasmids of strains are compared, has been used in the investigation of outbreaks caused by *E. faecalis* with high-level resistance to gentamicin (Huycke, Spiegel & Gilmore, 1991) and vancomycin-resistant *E. faecium* (Clark *et al*, 1993). However, this method can only be applied to strains containing plasmids. Furthermore, plasmids may be gained or lost from the strains and are therefore not stable markers.

DNA fingerprinting involves digesting genomic DNA with restriction endonucleases that have multiple recognition sites within the genome and then analysing the patterns of restricted fragments obtained after separation on agarose gels electrophoretically. However, large numbers of fragments are generated making interpretation difficult (Hall *et al*, 1992). This problem can be solved by using a probe which hybridizes with some of the fragments. A probe commonly used for this purpose is a rRNA probe. Its discriminatory power depends on the number and location of rRNA operons and the restriction enzymes used. Restriction enzymes such as *EcoR*I, *Hind*III and *Pst*I have been used for ribotyping of enterococcal strains (Gordillo, Singh & Murray, 1993; Bates, Jordens & Griffiths, 1994). Although hybridization patterns were found to be reproducible and relatively easy to interpret, ribotyping is time-consuming and laborious.

Another method to facilitate interpretation of DNA fragment patterns is to separate the fragments by pulsed-field gel electrophoresis (PFGE). PFGE separates DNA molecules by alternating electric fields (Birren & Lai, 1993; Kaufmann & Pitt, 1994; Tenover *et al*, 1995). Under the influence of the first electric field, DNA molecules elongate in the direction of the field and migrate into the gel. When the first field is removed and a second field is applied, at an angle to the first electric field, the DNA coil changes conformation and reorientates before migrating in the direction of the second field. Reorientation and separation is proportional to the size of DNA fragments. Numerous studies have used PFGE to separate restricted DNA fragments for typing of enterococci (Toye *et al*, 1997; Bonten *et al*, 1998; Fridkin *et al*, 1998; van den Braak *et al*, 1998 & 1999; Liassine *et al*, 1998). Various enzymes have been used but the most frequently used one is *Sma*I (Donabedian *et al*, 1995; Green *et al*, 1995; Reinert *et al*, 1999). Both contour-clamped homogenous electric field (CHEF) electrophoresis and field inversion gel electrophoresis (FIGE) can be used for PFGE. CHEF electrophoresis is performed in a special electrophoretic equipment (which is quite expensive) whereby alternating electric fields at 120° angle are provided while FIGE can be performed in a conventional electrophoretic chamber which provides inversion of electric current at 180° (Goering, 1993). The two techniques have been evaluated and shown to yield comparable results (Green *et al*, 1995). Despite the high cost in performing PFGE in CHEF equipment, it is more widely used, probably because of the better quality of the gel image and better separation of bands of >250 kb in size (Green *et al*, 1995).

PCR-based fingerprinting methods have also been used in the epidemiological studies of enterococci. The principle of this method is that when primers bind appropriately to specific regions of DNA of the test isolates, strain-specific amplification products may be obtained. Primers used may be specific such as in PCR-ribotyping where specific primers are used to amplify the 16S - 23S rRNA intergenic spacer region or non-specific as random amplified polymorphic DNA (RAPD) analysis whereby single primers of arbitrary sequence are used to anneal to multiple sites on the DNA under low-stringency conditions to produce a number of amplified DNA products (Williams *et al*, 1990; Welsh & McClelland, 1990; Castano-Anolles, Bassam & Gresshoff, 1991; Kostman *et al*, 1995; Barbier *et al*, 1996).

Prokaryotic genomes contain many families of highly repeated DNA sequences such as ERIC (enterobacterial repetitive intergenic consensus) sequences (Hulton, Higgins & Sharp, 1991), REP (repetitive extragenic palindromic) elements or palindromic units (PU) (Gilson *et al*, 1987) and BOX elements (Martin *et al*, 1992). Primers specific to these elements can be used in PCR for fingerprinting different bacterial species (Versalovic, Koeuth & Lupski, 1991). This particular type of PCR is known as repetitive sequence-based PCR or REP-PCR. Malathum and colleagues (1998) used REP-PCR to investigate the relatedness of *E. faecalis*. The primers used are based on the BOX elements which are interspersed repetitive DNA sequences found in *Streptococcus pneumoniae* (Martin *et al*, 1992). Both clonal and epidemiologically distant strains can be discriminated using this method.

However, results may not be reproducible and interpretation is often difficult, probably because fewer bands are generated and some products are not consistently visible (Malathum *et al*, 1998).

1.10. Epidemiological studies of enterococci

Most epidemiological studies of enterococci have concentrated on the investigation of outbreaks and dissemination of vancomycin-resistant enterococci while studies on the general epidemiology of enterococci are much less. PFGE has been applied to study the relatedness of β-lactamase-producing, high-level gentamicin resistant *E. faecalis* isolated in seven years from one hospital in the United States (Seetulsingh *et al*, 1996). Results showed that there was an endemic clone circulating in the hospital over the 7-year period. Studies in other parts of the United States also showed that spread of a limited number of penicillinase-producing strains are responsible for ampicillin-resistant enterococcal infections (Coudron, Markowitz & Wong, 1992; Seetulsingh *et al*, 1996). Bonten and colleagues (1996) used PFGE to demonstrate that colonization of the gastrointestinal tract or skin and contamination by the environment by VRE contribute to the spread of VRE in the hospital. Most VRE infections in hospitals are due to clonal spread of a single strain such as that found in South Africa (van Gottberg *et al*, 2000), Switzerland (Liassine *et al*, 1998) and Scotland (Nelson *et al*, 2000).

In contrast, Malathum *et al* (1998) found that of their 41 *E. faecalis* isolates studied, 17 are of distinct patterns and 24 can be classified into eight groups. Bischoff and colleagues (1999) found that four endemic strains are circulating in their hospital in Virginia, United States. Strains appeared and then disappeared after a certain period of time. They also found that more VREs are isolated in wards than in intensive care units (ICUs) which usually have a higher rate of isolation (Martone, 1998). Papaparaskevas *et al* (2000) also showed that there is considerable diversity amongst their high-level aminoglycoside-resistant (albeit vancomycin-sensitive) enterococcal strains. An interesting study by Suppola *et al* (1999) showed that both VRE and VSE (vancomycin-sensitive enterococci) isolated in their hospital are of the same type as demonstrated by PFGE. This is because VRE is the result of incorporation of *vanA* and *vanB* genes into an endemic VSE strain.

In the study by Issack, Power & French (1996), RAPD was used in the typing of vancomycin-resistant *E. faecium* (VREF) collected from 103 patients over a four-year period in a London teaching hospital. The results suggest that although several different strains of VREF are involved, cross-infection with individual types does occur on some wards. In another study, VRE are collected from 10 hospitals in Cincinnati in Ohio during a 6-month period (Perlada, Smulan & Cushion, 1997). PFGE revealed that all of the *E. faecium* isolates with the VanB phenotype are of identical or closely related patterns. This suggests that there has been a clonal dissemination of a single strain of VanB *E. faecium*. Reinert *et al* (1999) showed

that other than the common intra-hospital spread of VRE, inter-hospital spread of VRE can also take place. Fridkin and colleagues (1998) also noted that a dominant VRE strain at a hospital in Massachusetts has spread to another hospital, affecting a large number of patients. Also, an interesting study by van den Braak *et al* (1998), on the epidemiology of VRE from patients and poultry products shows that VRE isolated from patients are different from those from animals. It also shows that spread of resistance genes borne on transposons may be more important as the route of dissemination of VRE than clonal spread of resistant strains.

Recently, many studies have been performed on the epidemiology of nosocomial enterococcal infections especially with the increase of VRE. The understanding of the sources and dissemination of VRE requires the use of accurate typing method and PFGE was shown to be the most discriminating typing technique and usually considered as the "standard" for the typing of VRE (Boyle *et al,* 1993; Clark *et al*, 1993; Barbier *et al*, 1996)

In summary, PFGE appears to be the most favourable method in terms of discriminatory power, reproducibility and simplicity of procedures for the epidemiological studies of enterococci.

1.11. Objectives

Despite the increasing importance of enterococci as nosocomial pathogens in many parts of the world, there has been no study on the prevalence, distribution and antimicrobial susceptibilities of enterococci in Hong Kong. This study therefore aimed to:

- Determine the susceptibility of enterococci isolated in the Prince of Wales Hospital to antimicrobial agents;
- Evaluate methods for the identification of different enterococcal species;
- Determine the mechanisms of antibiotic resistance using biochemical and molecular methods; and
- Determine the degree of genetic relatedness of multiply-resistant enterococci by analysis of restriction DNA fragments using pulsed-field gel electrophoresis.

2. Materials and Methods

2.1. Collection of isolates

All non-duplicate enterococcal isolates (the first isolate from each patient) obtained from blood culture, cerebrospinal fluid and body fluids during April, 1997 - March, 1998 and from urine and bile during April - June of 1997 and 1998 in the Prince of Wales Hospital were collected. Isolates were stored on nutrient agar slopes (Oxoid, Basingstoke, Hampshire, England) at ambient temperature and in bead system (PROTECT Technical Service, Heywood, Lancashire, UK) at -20°C. Bacteria were recovered from storage by subculturing on blood agar.

2.2. Quality control strains

The control strains used in this study were *E. faecium* AIB40 (*vanA* gene), *E. faecalis* ATCC 51299 (*vanB* gene), *E. gallinarum* AIB 39 (*vanC-1* gene), *E. casseliflavus* ATCC 25788 (*vanC-2* gene), *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213 and *S. aureus* 77040101 (AAC6'-APH2").

2.3. Identification of isolates to genus level

Isolates were identified to genus level by the following screening tests: gram staining, microscopic morphology, production of catalase, growth on and blackening of bile-esculin agar and growth in the presence of 6.5% NaCl.

2.4. Identification of isolates to species level

2.4.1. By commercially available biochemical method

Isolates were identified to species level using the API 20 Strep system (bioMerieux SA, Montalieu Vercieu, France) according to procedures recommended by the manufacturer. Briefly, colonies grown overnight on blood agar were harvested using a swab and suspended in 2 ml sterile distilled water to give a turbidity greater than that of a McFarland No. 4 standard (equivalent to 12 x 10^8 CFU/ml). The suspension was inoculated into wells of the strip and incubated at 37°C for 4 to 24 hours. Results were read after addition of appropriate reagents to the wells and were interpreted as described in the manufacturer's insert. A seven-digit code was generated and the identification result was read off from the API 20 Strep Database (Version 6.0).

2.4.2. By molecular method - multiplex PCR

2.4.2.1. Source of primers

Four clinically relevant enterococci: *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus/E. flavescens* were identified by the multiplex PCR assay using four sets of oligonucleotide primers (Gibco BRL, Gaithersburg, MD, USA) based on published sequences (Dutka-Malen, Evers & Courvalin, 1995). Their nucleotide sequences are listed in Table 1.

Table 1. Oligonucleotide primers used in PCR for species identification of enterococci.

Amplified gene	Oligonucleotides		Position	PCR product size (bp)
	Pair	Nucleotide sequence, 5' to 3'		
$ddl_{E.faecalis}$	Entc9	+ATCAAGTACAGTTAGTCT	98-116	941
	Entc10	-ACGATTCAAAGCTAACTG	1038-1021	
$ddl_{E.faecium}$	Entc11	+TAGAGACATTGAATAATGCC	NA*	50
	Entc12	-TCGAATGTGCTACAATC	NA*	
vanC-1	Entc13	+GGTATCAAGGAAACCTC	246-272	822
	Entc14	-CTTCCGCCATCATAGCT	1067-1051	
vanC-2, *vanC-3*	Entc15	+CTCCTACGATTCTCTTG	455-486	439
	Entc16	-CAGGCAAGACCTTTAAG	885-869	

* NA = Not available

2.4.2.2. Extraction of bacterial DNA

Two to four overnight isolated colonies on blood agar were selected and suspended in 500 µl of sterile distilled water. The suspension was heated at 94°C for 8 minutes using a heating block (TRU TEMP™ DNA micro-heating system, Robbins Scientific, Sunnyvale, CA., USA), centrifuged at 4,000 g (Biofuge 22R, Heraeus Sepatech, Osterode, Germany) for 5 minutes and DNA in the supernatant was used as template for PCR.

2.4.2.3. Multiplex PCR

The 25 µl-volume PCR mixture contained sterile distilled water, 1x PCR buffer, 0.2 mM of dNTP, 2 mM of $MgCl_2$, 0.5 µM each of the eight primers, 0.5 unit of *Taq* polymerase (all from Gibco) and 3 µl of template DNA. DNA amplification was performed on a thermal cycler (PTC-100™ Programmable Thermal Controller, MJ Research Inc., Watertown, MA, USA) according to the following protocol: denaturation at 94°C for 2 minutes, then 30 cycles at 94°C for 60s, 53°C for 60s and 72°C for 60s, and a final extension reaction at 72°C for 10 minutes.

2.4.2.4. Analysis of PCR products by agarose gel electrophoresis

PCR products (5µl) with 1µl of 6x loading buffer (0.25 % bromophenol blue and 15 % Ficoll type 400, Sigma) were subjected to electrophoresis (GIBCO BRL Horizontal gel electrophoresis apparatus) on 1.5% agarose gel (Sigma) in 1x Tris-borate-EDTA (TBE) buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA,

Sigma) at 70V for 1 hour. The molecular size marker used was *Hae*III-digested ϕx174 phage DNA (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The gel was stained by ethidium bromide (1 μg/ml) for 15 minutes and washed with distilled water. It was visualized under ultraviolet (UV) illumination and was photographed using a gel documentation system (Image Master VDS, Pharmacia).

2.4.3. By conventional biochemical methods

Isolates giving discrepant results using the two identification methods were re-identified by conventional biochemical methods according to the published scheme of Facklam and Collins (1989). The tests performed included acidification of 1% mannitol, sorbitol, arabinose and raffinose in heart infusion broth (Difco, Detroit, MI, USA) with bromocresol purple (BDH, Poole, England) as the indicator, deamination of arginine in Moellers decarboxylase medium (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and formation of yellow pigment after overnight incubation on trypticase soya agar (Oxoid). Motility was determined by motility testing medium (Difco). All tests were incubated at 37°C except for motility that was incubated at 30°C for up to five days.

2.5. Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of different antimicrobial agents were determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1997). The following drugs were tested: penicillin (P), ampicillin (A), gentamicin (G),

streptomycin (St), erythromycin (E), trimethoprim (Tm), sulfamethoxazole, chloramphenicol (C), rifampicin (Rif), sparfloxacin (Spx) (RP64206) from Sigma Chemical Co., St Louis, Mo., USA, vancomycin (V) (Abbott Labs, North Chicago, IL, USA), teicoplanin (Tei) (Marion Merrell Dow, Cincinnati, OH, USA) and quinupristin/dalfopristin (RP59500) (Rhône-Poulenc Rorer, Cologne, Germany).

2.5.1. Preparation of antimicrobial agent stock solution

The following formula was used to determine the amount of antibiotic powder and volume of diluent of an antibiotic stock solution:

$$\text{Weight of antibiotic (mg)} = \frac{\text{Volume of diluent (ml)} \times \text{Concentration of stock solution (}\mu\text{g/ml)}}{\text{Potency of antibiotic (}\mu\text{g/mg)}}$$

The antimicrobial stock solutions were aliquoted into sterile polypropylene or polyethylene vials and stored at -60°C or below.

2.5.2. Preparation of agar medium

Mueller-Hinton agar (Oxoid) was used except for testing trimethoprim and co-trimoxazole when Mueller-Hinton agar with 5% lysed horse blood was used. Appropriate volumes of serial two-fold dilutions of antibiotics were added to molten agar to give the final concentrations. The molten agar was then mixed thoroughly and poured into a petri dish on a leveled surface. The agar was allowed to solidify at room temperature and either used immediately (drugs such as β-lactams and tetracyclines which deteriorate rapidly) or stored in sealed plastic bags

at 4°C - 8°C for up to 5 days. The plates were equilibrated to room temperature before use. Drug-free Mueller-Hinton plates were used as controls.

2.5.3. Preparation of inoculum and inoculation of plates

The inoculum was prepared by emulsifying a few overnight colonies of a single type in 3 ml of normal saline. The turbidity was adjusted to match that of a 0.5 McFarland standard (equivalent to approximately 10^8 CFU/ml). The suspension was then diluted 10-fold with saline to give a concentration of 10^7 CFU/ml. Antibiotic and control plates were inoculated with this inoculum using a multipoint inoculator (MIC 2000, Dynatech Instrument, Inc., CA, USA) which delivered approximately 1 µl giving a final inoculum of 10^4 CFU/spot. The following control strains were included in each experiment: ATCC 29212 (*E. faecalis*), ATCC 51299 (*E. faecalis*), ATCC 25788 (*E. casseliflavus*), AIB40 (*E. faecium*), AIB39 (*E. gallinarum*) and ATCC 29213 (*S. aureus*).

2.5.4. Incubation and reading of plates

The inoculated plates were left at room temperature until moisture on the inoculation spots was completely absorbed into the agar. The plates were inverted and incubated at 37°C for 16 to 20 hours. The MIC was determined as the lowest concentration of antimicrobial agent that completely inhibited visible growth at the inoculation spot.

The percentage of resistance was determined by the breakpoint criteria used to define susceptible strains based on the NCCLS MIC interpretative standards (1999) except for trimethoprim and co-trimoxazole (Sxt) when interpretative standards for urinary tract infection were used (≤ 8 mg/L and ≤ 32 mg/L, respectively). NCCLS did not define any MIC interpretative standard for sparfloxacin so a breakpoint of 1 mg/L (similar to that of ciprofloxacin) was used.

2.6. Mechanisms of antibiotic resistance

2.6.1. Amplification of vancomycin resistance genes in enterococci by multiplex PCR

2.6.1.1. Isolates

Enterococci with vancomycin MIC ≥ 4mg/L were selected for PCR. The following strains were used as controls: *E. faecium* AIB40 (*vanA* gene), *E. faecalis* ATCC 51299 (*vanB* gene), *E. gallinarum* AIB 39 (*vanC-1 gene*), *E. casseliflavus* ATCC 25788 (*vanC-2* gene) and *E. faecalis* ATCC 29212 (vancomycin sensitive strain).

2.6.1.2. Primers

The oligonucleotide primers (Gibco) used were as described by Dutka-Malen, Evers and Courvalin (1995) and Free and Sahm (1995) and are listed in Table 2. Acquired vancomycin resistance genotypes were identified by specific amplification of the *vanA* and *vanB* genes and intrinsic vancomycin resistance genotypes by specific amplification of the *vanC-1* and *vanC-2/C-3* genes.

2.6.1.3. Extraction of bacterial DNA and PCR

These were performed as described in sections 2.4.2.2. and 2.4.2.3. except that the amplification conditions were as follows: denaturation at 95°C for 3 minutes, 30 cycles of 94°C for 60s, 56°C for 60s and 72°C for 60s, and a final extension at 72°C for 10 minutes. PCR products were detected by agarose gel electrophoresis as described in section 2.4.2.4. Two PCRs were set up for each sample. One contained primer pairs for the detection of *vanA* and *vanB* genes (783 and 297 bp PCR fragments, respectively) and the other contained primer pairs for the detection of *vanC-1* and *vanC-2* or *vanC-3* genes (822 and 439 bp PCR fragments, respectively).

Table 2. Oligonucleotide primers for the amplification of vancomycin resistance genes.

Amplified Gene	Oligonucleotide		Size of PCR product (bp)
	Pair	Nucleotide sequence, 5' to 3'	
vanA	VanA1	+GCTATTCAGCTGTACTC	783
	VanA2	-CAGCGGCCATCATACGG	
vanB	VanB1	+CATCGCCGTCCCCGAATTTCAAA	297
	VanB2	-GATGCGGAAGATACCGTGGCT	
vanC-1	VanC1-1	+GGTATCAAGGAAACCTC	822
	VanC1-2	-CTTCCGCCATCATAGCT	
vanC-2/3	VanC2-1	+CTCCTACGATTCTCTTG	439
	VanC2-2	-CGAGCAAGACCTTTAAG	

2.6.2. Detection of gene coding for aminoglycoside-modifying enzyme (AAC6'/APH2") by PCR

2.6.2.1. Isolates

All enterococci with gentamicin MIC ≥1024mg/L were subjected to PCR. S. *aureus* ATCC 29213 was used as a negative control and *S. aureus* 77040101 which produced AAC6'/APH2" was used as a positive control. Twenty strains with gentamicin MIC ≤256 mg/L were also selected randomly for testing.

2.6.2.2. Primers

The oligonucleotide primers (Gibco) used were as reported by van de Klundert and Vliegenthart (1993) and are listed in Table 3. The 16S rRNA primers were used as internal control and absence of the rRNA amplified signals indicated failure of the PCR.

2.6.2.3. Extraction of bacterial DNA and PCR

These were performed as described in sections 2.4.2.2. and 2.4.2.3. except that the reaction mixture and PCR conditions were as follows: 25µl-volume PCR mixture contained sterile distilled water, 1x PCR buffer, 0.2 mM of dNTP, 2 mM of $MgCl_2$, 0.6 µM of four primers, 0.5 unit of *Taq* polymerase and 2 µl of template DNA; denaturation at 95°C for 3 minutes, 32 cycles of 94°C for 60s, 56°C for 60s and 72°C for 60s, and a final extension at 72°C for 10 minutes. PCR products were detected by agarose gel electrophoresis as described in section 2.4.2.4.

Table 3. Oligonucleotide primers for amplification of gene coding for the aminoglycoside modifying enzyme AAC6'/APH2".

Amplified Gene	Oligonucleotide		Position	Size of PCR product (bp)
	Pair	Sequence, 5' to 3'		
16S rRNA	Rrs1	+GGATTAGATACCCTGGTAGTCC	775-796	320
	Rrs2	-TCGTTGCGGGACTTAACCCAAC	1095-1074	
aacA/aphD (AAC6'/APH2'')	Ame1	+CCAAGAGCAATAAGGGCATA	423-442	220
	Ame2	-CACTATCATAACCACTACCG	643-624	

2.6.3. Detection of β-lactamase in enterococci

Enterococci with ampicillin MICs >8 mg/L were screened for the presence of β-lactamase with cefinase disc (BBL Microbiology Systems, Cockeysville, MD, USA) according to the manufacturer's instruction. Briefly, the disc was moistened with distilled water and a few colonies were placed on it. A yellow to red colour change within 5 minutes on the area where the culture was applied indicated a positive reaction and the presence of β-lactamase.

2.7. To study the genetic relatedness of multi-resistant enterococci by pulsed-field gel electrophoresis (PFGE)

Multi-resistant enterococci were selected for molecular typing by pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared as described (Murray, 1990; Donabedian *et al*, 1995; Vandamme *et al*, 1996; Perlada, Smulian & Cushion, 1997; Toye *et al*, 1997) but with modifications. All reagents used were from Sigma Chemical Co. unless indicated otherwise.

2.7.1. Incorporation of chromosomal DNA into agarose plugs

About half a loopful of overnight colonies on blood agar was suspended in 500 µl SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5). Cells were pelleted by centrifuging at 4,000 g for 10 minutes, washed three times with SE buffer and finally resuspended in 500 µl SE buffer. Equal volumes of bacterial suspension and 2% Seaplaque GTG low-melting-agarose (FMC, Rockland, MA, USA) in SE buffer at 56°C were mixed. The mixture was dispensed into plug moulds (Bio-Rad,

Hercules, CA, USA) and kept at 4°C for at least 30 minutes or until the agarose was set. The plugs were incubated overnight at 37°C in 3 ml of lysis buffer (6 mM Tris HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, 1 mg of lysozome per ml), replaced by 3 ml of proteolysis buffer (0.5 M EDTA-pH 9.5, 1% N-lauroyl sarcosine and 100 µg/ml of proteinase K (GIBCO BRL)) and were incubated for a further 24 hours at 56°C. The plugs were then washed three times with 5 ml of TE buffer at 4°C and used immediately or stored at 4°C in TE buffer.

2.7.2. Digestion of chromosomal DNA in agarose plugs with restriction enzyme *Sma*I

A portion of a plug was cut and placed in an Eppendorf tube containing 100 µl restriction buffer (1x, supplied with the restriction enzyme). The plug was kept at 4°C for 2 hours. The buffer was replaced with fresh restriction buffer (1x) containing 20 IU of *Sma*I (Amersham Pharmacia) and was incubated for 16-20 hours at 30°C.

2.7.3. Pulsed-field gel electrophoresis

The plugs were loaded into wells of a 1.2% agarose gel (Seakem gold agarose, FMC) in 0.5x TBE buffer and electrophoresed on a pulsed-field gel electrophoresis equipment (CHEF MAPPER, Bio-Rad). The parameters for electrophoresis were 6 V/cm for 20 hours, pulse times ramped from 5 to 35 s, ramping factor: linear, included angle: 120° and temperature was 14°C.

Bacteriophage λ DNA-PFGE markers (Amersham Pharmacia) were used as size marker. After electrophoresis, the gels were stained in ethidium bromide, washed in distilled water and photographed under UV illumination by a gel documentation system (Image Master VDS, Pharmacia).

2.7.4. Cluster analysis

The PFGE patterns were compared by the Dice coefficient (Arbeit, 1999) and cluster analysis performed by the unweighted pair group method with arithmetic average (Sneath, 1972) using the GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium). Patterns with a Dice coefficient of ≥0.9 (i.e. of ≥90% similarity) were highly similar and likely to be epidemiologically related (Arbeit, 1999). These patterns were therefore grouped as a single group.

2.7.5. Date analysis

The chi square test was used for statistical analysis.

2.8. Research plan

All *Enterococcus* species isolated from urine and bile during the three-month period (April to June) in 1997 and 1998 and from blood, body fluids and cerebrospinal fluid from April 1997 to March 1998 collected from patients in the Prince of Wales Hospital and which survived were included in the study.

1. Evaluation of polymerase chain reaction (PCR) for the identification of enterococci.

All enterococci isolated in 1997 were identified by PCR and API 20 Strep and the identities confirmed by biochemical tests.

2. Identification of all enterococcal isolates.

Isolates subsequently collected in 1998 were identified using PCR only and all non-*E. faecalis* isolates were re-identified by biochemical tests.

3. Antimicrobial susceptibility testing.

The agar dilution method (NCCLS, 1997) was used to determine minimal inhibitory concentrations (MICs) of 13 antimicrobial agents to *Enterococcus* species.

4. Mechanisms of antibiotic resistance.

Isolates resistant to vancomycin (MIC ≥ 4mg/L) or high-levels of gentamicin (MIC ≥1024 mg/L) were subjected to PCR for detection of *van* or *aac(6')-aph(2")* genes, respectively. Isolates resistant to ampicillin (MIC >8mg/L) were tested for the production of β-lactamase.

5. Epidemiology of enterococci.

Multi-resistant enterococcal isolates were typed by pulsed-field gel electrophoresis (PFGE) and restriction patterns analyzed using the GelCompar program.

3. Results

A total of 498 non-duplicate isolates obtained from April to June in 1997 from urine and bile and April in 1997 to March in 1998 from blood, body fluids and cerebrospinal fluid (CSF) were studied. These included 49 isolates from blood cultures, six from body fluids (four from ascitic fluid and two from pleural fluid), two from CSF, 44 from bile (26 in 1997 and 18 in 1998) and 397 from urine (225 in 1997 and 172 in 1998).

3.1. Identification of enterococci by API 20 Strep and PCR

Three hundred and nine isolates from urine and bile in 1997 and blood cultures, body fluids and CSF from 1997 to 1998 were identified to species level using API 20 Strep, PCR and conventional tests. The remaining 189 isolates were identified by multiplex PCR supplemented by conventional tests.

Two hundred and ninety-eight strains were identified with an excellent to good API identification score using API 20 Strep system (Table 4). These were *E. faecalis* (246), *E. faecium* (47), *E. avium* (2), *E. casseliflavus* (2) and *E. gallinarum* (1). However, 11 strains could only be identified to genus level. The identification ranged from low discrimination to good. These included *E. faecium* or *E. gallinarum* (3), *E. gallinarum* or *E. faecalis* (2), *E. gallinarum* or *E. faecium*, *E. faecalis* or *E. durans*, *E. durans* or *E. faecium*, *E. faecalis* or *E. faecium* (1 each) and *E. avium* or *Aerococcus* (2).

Table 4. Identification of enterococci by API 20 Strep

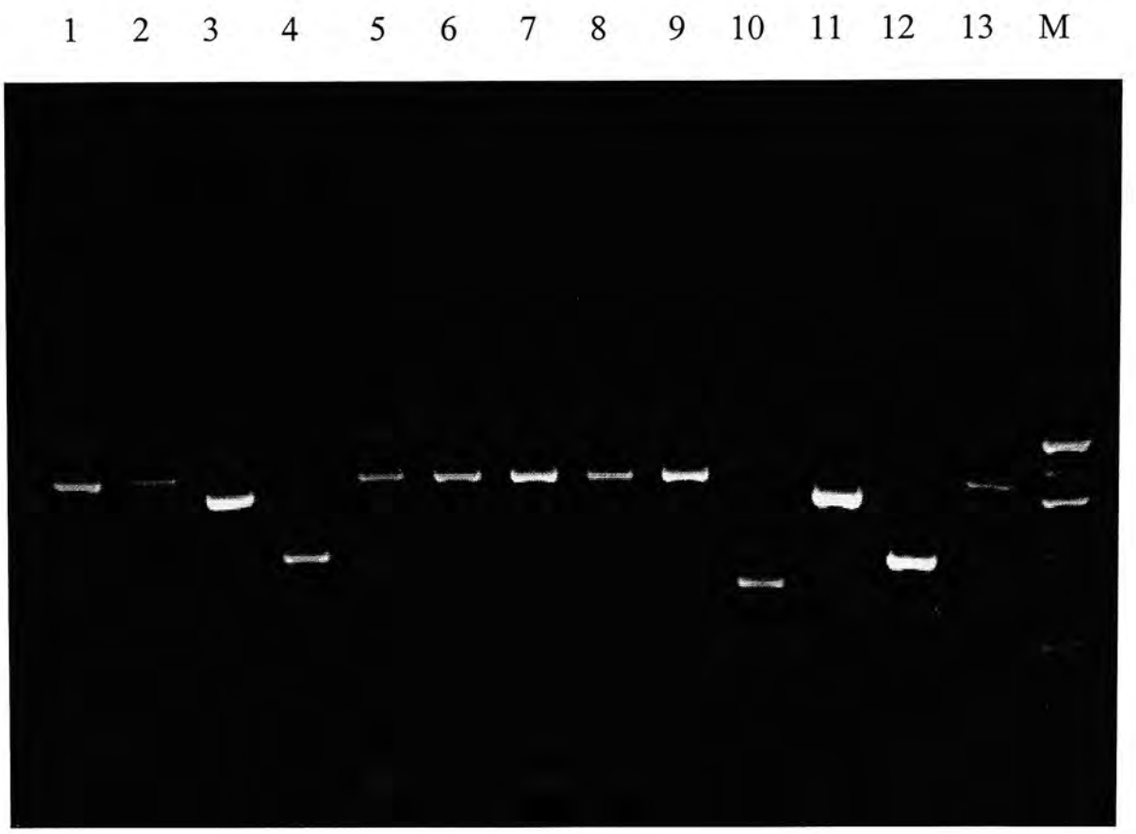
API identification score	API identification	No. of strains (%)
Excellent to good	*E. faecalis*	246 (82.5)
	E. faecium	47 (15.8)
	E. avium	2 (0.7)
	E. casseliflavus	2 (0.7)
	E. gallinarum	1 (0.3)
	Total	**298 (100)**
Low discrimination/ good to genus level only	*E. faecalis/ E. faecium*	1 (9.1)
	E. faecalis/ E. durans	1 (9.1)
	E. faecium/ E. gallinarum	3 (27.2)
	E. gallinarum/ E. faecalis	2 (18.2)
	E. gallinarum/ E. faecium	1 (9.1)
	E. durans/ E. faecium	1 (9.1)
	E. avium/ Aerococcus	2* (18.2)
	Total	**11 (100)**

*: the two isolates identified as either *E. avium* or *Aerococcus* were finally confirmed to be *E. avium* due to their ability to grow at 45°C.

The API Database suggested several additional tests for definite identification of these isolates. For example, formation of yellow pigment was used to differentiate *E. casseliflavus* from *E. faecium* and *E. gallinarum*. Growth at 45°C was used to differentiate *E. avium* from *Aerococcus*: *E. avium* could grow at 45°C while *Aerococcus* could not. By this test, the two isolates that were identified as *E. avium* or *Aerococcus* were finally identified as *E. avium*. For the other strains, either no complementary test was suggested by the API Database or the suggested tests were not available in our laboratory. Thus, their final identifications were according to the results suggested by API Database.

Three hundred and three isolates could be identified to species level by multiplex PCR. PCR products of the expected sizes (Table 1) were obtained (Figure 1). Two hundred and forty-three isolates were identified as *E. faecalis*, 52 as *E. faecium*, five as *E. gallinarum* and three as *E. casseliflavus*. Six isolates could not be identified as no amplified product was produced after PCR. This indicated that they were probably enterococcal species other than *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*.

Figure 1. Agarose gel electrophoresis of amplified products after multiplex PCR using species-specific primers for enterococci.



Amplified products from isolates were contained in lanes 1 – 9. Lanes 1, 2, 5 - 9: *E. faecalis*, Lane 3: *E. gallinarum*, Lane 4: *E. faecium*. Amplified product from control strains were contained in lanes 10 –13. Lane 10: *E. casseliflavus* (ATCC 25788), Lane 11: *E. gallinarum* (AIB39), Lane 12: *E. faecium* (AIB40), Lane 13: *E. faecalis* (ATCC 51299). Size marker (ϕX174 phage DNA - *Hae*III digest) in lane M.

Both API 20 Strep and PCR gave the same results for 287 isolates (Table 5). These included 240 *E. faecalis* isolates, 45 *E. faecium* isolates and one isolate each of *E. gallinarum* and *E. casseliflavus*. Their identities were confirmed by conventional tests except for those identified as *E. faecalis*. Twenty-two isolates gave discrepant results. To confirm the identities of these 22 isolates, acid formation of sorbitol and arabinose broths, motility and formation of yellow pigment were performed (Facklam & Collins, 1989) (Table 5). For *E. durans*, deamination of arginine and acid formation of mannitol and arabinose broth were performed and for *E. avium*, deamination of arginine and acid formation of mannitol, sorbitol, arabinose and raffinose broths were performed (Table 5). Five isolates that were identified as *E. faecalis* by API were finally identified as *E. faecium* because of its ability to form acid from arabinose broth but not from sorbitol broth. One isolate that was identified as *E. faecalis* by API was confirmed to be *E. gallinarium* because of its ability to form acid from arabinose broth but not from sorbitol broth, motility and absence of pigmentation. One isolate that was identified as either *E. faecalis* or *E. faecium* was finally identified as *E. faecalis* due to its ability to form acid from both arabinose and sorbitol broths.

Table 5. Comparison of API 20 Strep, PCR and conventional method for the identification of 309 isolates of enterococci.

	Identification results by			
API 20 Strep	PCR	Conventional method	Final ID	No.
E. faecalis	*E. faecalis*	ND	*E. faecalis*	240^
E. faecalis	*E. faecium*	*E. faecium* (Sor- Ara+)	*E. faecium*	5*
E. faecalis	*E. gallinarum*	*E. gallinarum* (Sor- Ara+ Mot+ Pig-)	*E. gallinarium*	1*
E. faecalis/ E. faecium	*E. faecalis*	*E. faecalis* (Sor+ Ara-)	*E. faecalis*	1*
E. faecalis/ E. durans	Negative	*E. durans* (Arg+ Man- Sor- Ara- Raf-)	*E. durans*	1
E. faecium	*E. faecium*	*E. faecium* (Sor- Ara+)	*E. faecium*	45^*
E. faecium	*E. faecalis*	*E. faecalis* (Sor+ Ara-)	*E. faecalis*	2*
E. gallinarum	*E. gallinarum*	*E. gallinarum* (Sor- Ara+ Mot+ Pig-)	*E. gallinarum*	1^*
E. gallinarum/ E. faecalis	*E. gallinarum*	*E. gallinarum* (Sor- Ara+ Mot+ Pig-)	*E. gallinarum*	1*
E. gallinarum/ E. faecalis	*E. faecium*	*E. faecium* (Sor- Ara+ Mot- Pig-)	*E. faecium*	1*
E. gallinarum/ E. faecium	*E. faecium*	*E. faecium* (Sor- Ara+ Mot- Pig-)	*E. faecium*	1*
E. gallinarum/ E. faecium	*E. gallinarum*	*E. gallinarium* (Sor- Ara+ Mot+ Pig-)	*E. gallinarum*	1*
E. gallinarum/ E.faecium	*E. casseliflavus*	*E. casseliflavus* (Sor- Ara+ Mot+ Pig-)	*E. casseliflavus*	2*
E. casseliflavus	*E. casseliflavus*	*E. casseliflavus* (Sor- Ara+ Mot+ Pig-)	*E. casseliflavus*	1^*
E. casseliflavus	*E. gallinarum*	*E. gallinarum* (Sor- Ara+ Mot+ Pig-)	*E. gallinarum*	1*
E. avium	Negative	*E. raffinosus* (Arg- Man+ Sor- Ara+ Raf+)	*E. raffinosus*	2
E. avium/ Aerococcus	Negative	*E. raffinosus* (Arg- Man+ Sor- Ara+ Raf+)	*E. raffinosus*	1
E. avium/ Aerococcus	Negative	*E. avium* (Arg- Man+ Sor- Ara+ Raf-)	*E. avium*	1
E. durans/ E. faecium	Negative	*E. durans* (Arg+ Man- Sor- Ara- Raf-)	*E. durans*	1
			Total	309

(Ara, arabinose; Arg, arginine; Man, mannitol; Raf, raffinose; Sor, sorbitol; Mot, motility; Pig, pigment production; ND, not done; ^, same results for API 20 Strep and PCR; *, same results for PCR and conventional method)

The conventional tests gave the same identification results as PCR for 16 of these 22 isolates which yielded discrepant results by API 20 Strep and PCR. The remaining six isolates were found to be species other than *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus* since no amplification products were obtained after PCR. Thus, PCR was chosen as the method for identification of all isolates from urine and bile collected in 1998 and the identity of those which were shown to be non-*E. faecalis* by PCR was confirmed by the conventional scheme.

Among the 498 isolates collected from 1997 to 1998, 403 (81%) were *E. faecalis*, 77 (15%) were *E. faecium* and 18 (4%) were other *Enterococcus spp.* (eight of *E. gallinarum*, four of *E. casseliflavus*, three of *E. raffinosus*, two of *E. durans* and one of *E. avium*) (Table 6). A similar proportion of *E. faecalis* isolates were isolated from urine and bile during the two years: 86% (193 of 225) in 1997 and 87% (149 of 172) in 1998 from urine; 46% (12 of 26) in 1997 and 67% (12 of 18) in 1998 from bile. Similarly, the same proportion of *E. faecium* isolates were collected from urine during the two years, (13% (30 of 225) in 1997 and 13% (22 of 172) in 1998). More *E. faecium* isolates were collected from bile collected in 1997 than in 1998 (31% (8 of 26) and 16% (3 of 18), respectively). The number of enterococcal species collected from blood, body fluids and CSF was small (49 from blood, six from body fluids and two from CSF). The majority of these isolates were *E. faecalis* (65%) while *E. faecium* made up 25%.

Table 6. Distribution of enterococcal species from different specimens

Organism	Urine		Bile		Blood	Body fluid	CSF	Total (%)
	1997	1998	1997	1998	1997-98	1997-98	1997-98	
E. faecalis	193	149	12	12	32	3	2	403 (80.9)
E. faecium	30	22	8	3	12	2		77 (15.5)
E. gallinarum	1		2	3	2			8 (1.6)
E. casseliflavus		1	1		1			4 (0.8)
E. durans			1		1			2 (0.4)
E. avium						1		1 (0.2)
E. raffinosus	1		1		1			3 (0.6)
Sub-total	225	172	26	18				
Total (%)	397 (79.7)		44 (8.8)		49 (9.8)	6 (1.2)	2 (0.4)	498 (100)

3.2. Antimicrobial susceptibilities of enterococci

The susceptibilities of enterococci to 13 antimicrobial agents are summarized in Table 7. The MIC range of penicillin and ampicillin for *E. faecalis* was similar, being 0.5 or 1 mg/L - >16 mg/L. The MIC_{50} and MIC_{90} of these two drugs were also similar, being 1- 2 mg/L. Only three and two (1% each) of the 404 *E. faecalis* isolates tested were resistant to penicillin and ampicillin, respectively. However, 95% of the 76 *E. faecium* isolates tested were resistant to penicillin and ampicillin. This was highly significant ($p < 0.01$). The MIC range of penicillin and ampicillin for *E. faecium* was the same (1 - >16 mg/L) and MIC_{50} and MIC_{90} were both >16mg/L.

None of the *E. faecalis* isolates was resistant to vancomycin (MIC range 0.5 – 4 mg/L; MIC_{50} = 1 mg/L; MIC_{90} = 2 mg/L) and teicoplanin (MIC range 0.03 – 0.5 mg/L; MIC_{50} = 0.12 mg/L; MIC_{90} = 0.25 mg/L). There was only one *E. faecium* isolate that was resistant to both vancomycin and teicoplanin (MIC >16 mg/L) and one to vancomycin (MIC >16 mg/L) only. These two strains were isolated from urine in 1998. The MIC range of vancomycin for the vancomycin-sensitive *E. faecium* was 0.5 – 4 mg/L and that of teicoplanin was 0.06 – 0.5 mg/L.

The MIC range of gentamicin for both *E. faecalis* and *E. faecium* was similar, being 2 or 4 mg/L - >1024 mg/L. High-level resistance to gentamicin (MIC >512 mg/L) was detected in 106 (26%) *E. faecalis* isolates and 16 (21%) *E. faecium* isolates. Significantly more *E. faecalis* and *E. faecium* strains isolated in 1998 (34% and 31%, respectively) were resistant to high-level gentamicin than

Table 7. Antimcrobial susceptibilities of *Enterococcus* species isolated from 1997 to 1998.

Antibiotic	*Enterococcus* spp.	Year	Site	No.	MIC Range	MIC$_{50}$	MIC$_{90}$	% of R'	Bp*
Penicillin (P)	*E. faecalis*	1997	all	205	1 - >16	2	2	2	8
		1998	all	198	1 - >16	2	4	0	
		1997-98	urine	342	1 - >16	2	2	1	
		1997-98	bile	24	1 - >16	2	2	1	
		1997-98	blood	32	1 - 4	2	4	0	
		Total		403	1 - >16	2	2	1	
	E. faecium	1997	all	38	1 - >16	>16	>16	95	
		1998	all	39	2 - >16	>16	>16	95	
		1997-98	urine	52	>16	>16	>16	100	
		1997-98	bile	11	1 - >16	>16	>16	64	
		1997-98	blood	12	>16	>16	>16	100	
		Total		77	1 - >16	>16	>16	95	
	Others	1997-98	all	18	0.5 - 16	1	16	11	
	Total	1997-98		498	0.5 - >16	2	>16	16	
Ampicillin (A)	*E. faecalis*	1997	all	205	0.5 - >16	1	1	1	8
		1998	all	198	0.5 - >16	1	2	0	
		1997-98	urine	342	0.5 - >16	1	2	1	
		1997-98	bile	24	0.5 - >16	2	2	4	
		1997-98	blood	32	0.2 - 2	1	2	0	
		Total		403	0.5 - >16	1	2	1	
	E. faecium	1997	all	38	0.5 - >16	>16	>16	78	
		1998	all	39	2 - >16	>16	>16	95	
		1997-98	urine	52	>16	>16	>16	100	
		1997-98	bile	11	1 - >16	>16	>16	64	
		1997-98	blood	12	>16	>16	>16	100	
		Total		77	1 - >16	>16	>16	87	
	Others	1997-98	all	18	0.5 - >16	1	8	6	
	Total	1997-98		498	0.5 - >16	1	>16	15	
Vancomycin (V)	*E. faecalis*	1997	all	205	0.5 - 4	1	2	0	4
		1998	all	198	0.5 - 4	2	4	0	
		1997-98	urine	342	0.5 - 4	1	2	0	
		1997-98	bile	24	0.5 - 4	2	4	0	
		1997-98	blood	32	1 - 4	1	2	0	
		Total		403	0.5 - 4	1	2	0	
	E. faecium	1997	all	38	0.5 - 4	1	2	0	
		1998	all	39	0.5 - >16	1	2	5	
		1997-98	urine	52	0.5 - >16	1	1	4	
		1997-98	bile	11	0.5 - 1	1	1	0	
		1997-98	blood	12	0.5 - 1	1	1	0	
		Total		77	0.5 - >16	1	1	3	
	Others	1997-98	all	18	0.25 - 8	4	8	11	
	Total	1997-98		498	0.25 - >16	1	2	1	

(To be continued)

Table 7. Antimcrobial susceptibilities of *Enterococcus* species isolated from 1997 to 1998.

Antibiotic	*Enterococcus* spp.	Year	Site	No.	MIC Range	MIC$_{50}$	MIC$_{90}$	% of R'	Bp*
Teicoplanin (Tei)	*E. faecalis*	1997	all	205	0.03 - 0.5	0.12	0.25	0	8
		1998	all	198	0.03 - 0.5	0.12	0.25	0	
		1997-98	urine	342	0.03 - 0.5	0.12	0.25	0	
		1997-98	bile	24	0.06 - 0.5	0.12	0.25	0	
		1997-98	blood	32	0.06 - 0.5	0.12	0.5	0	
		Total		403	0.03 - 0.5	0.12	0.25	0	
	E. faecium	1997	all	38	0.06 - 0.5	0.25	0.5	0	
		1998	all	39	0.12 - >16	0.5	0.5	3	
		1997-98	urine	52	0.12 - 0.5	0.25	0.5	2	
		1997-98	bile	11	0.06 - 0.5	0.25	0.5	0	
		1997-98	blood	12	0.12 - 0.5	0.25	0.5	0	
		Total		77	0.06 - >16	0.5	0.5	1	
	Others	1997-98	all	18	0.06 - 0.5	0.25	0.5	0	
	Total	1997-98		498	0.03 - 32	0.12	0.5	0.2	
Gentamicin (G)	*E. faecalis*	1997	all	205	4 - >1024	32	>1024	19	512
		1998	all	198	4 - >1024	64	>1024	34	
		1997-98	urine	342	4 - >1024	32	>1024	27	
		1997-98	bile	24	4 - >1024	32	1024	26	
		1997-98	blood	32	8 - >1024	128	>1024	25	
		Total		403	4 - >1024	32	>1024	26	
	E. faecium	1997	all	38	2 - >1024	8	>1024	11	
		1998	all	39	4 - >1024	16	>1024	32	
		1997-98	urine	52	4 - >1024	16	>1024	18	
		1997-98	bile	11	4 - 1024	8	1024	9	
		1997-98	blood	12	4 - >1024	16	>1024	42	
		Total		77	4 - >1024	16	>1024	21	
	Others	1997-98	all	18	4 - >1024	8	>1024	11	
	Total	1997-98		498	4 - >1024	32	>1024	25	
Streptomycin (St)	*E. faecalis*	1997	all	205	4 - >1024	256	>1024	38	>1024
		1998	all	198	8 - >1024	256	>1024	43	
		1997-98	urine	342	4 - >1024	256	>1024	33	
		1997-98	bile	24	64 - >1024	1024	>1024	46	
		1997-98	blood	32	32 - >1024	256	>1024	59	
		Total		403	4 - >1024	256	>1024	36	
	E. faecium	1997	all	38	32 - >1024	>1024	>1024	74	
		1998	all	39	32 - >1024	>1024	>1024	62	
		1997-98	urine	52	32 - >1024	>1024	>1024	73	
		1997-98	bile	11	32 - >1024	64	>1024	46	
		1997-98	blood	12	128 - >102	>1024	>1024	67	
		Total		77	32 - >1024	>1024	>1024	67	
	Others	1997-98	all	18	32 - >1024	32	>1024	17	
	Total	1997-98		498	4 - >1024	256	>1024	40	

(To be continued)

Table 7. Antimcrobial susceptibilities of *Enterococcus* species isolated from 1997 to 1998.

Antibiotic	*Enterococcus* spp.	Year	Site	No.	MIC Range	MIC$_{50}$	MIC$_{90}$	% of R'	Bp*
Chloramphenicol (C)	*E. faecalis*	1997	all	205	4 - 64	8	32	41	8
		1998	all	198	4 - 64	8	64	46	
		1997-98	urine	342	4 - 64	8	32	43	
		1997-98	bile	24	4 - 64	8	32	38	
		1997-98	blood	32	4 - 64	8	32	44	
		Total		403	4 - 64	8	32	44	
	E. faecium	1997	all	38	0.5 - 32	8	16	28	
		1998	all	39	1 - 32	8	32	31	
		1997-98	urine	52	1 - 32	4	16	17	
		1997-98	bile	11	0.5 - 32	8	8	9	
		1997-98	blood	12	8 - 32	4	16	17	
		Total		77	4 - 64	8	32	29	
	Others	1997-98	all	18	0.25 - 8	4	32	22	
	Total	1997-98		498	2 - 64	8	32	41	
Rifampicin (Rif)	*E. faecalis*	1997	all	205	0.25 - >16	2	8	59	1
		1998	all	198	0.25 - >16	2	8	55	
		1997-98	urine	342	0.25 - >16	2	8	57	
		1997-98	bile	24	0.25 - 8	1	4	50	
		1997-98	blood	32	1 - >16	4	8	59	
		Total		403	0.25 - >16	2	8	56	
	E. faecium	1997	all	38	0.5 - >16	8	>16	86	
		1998	all	39	1 - >16	>16	>16	97	
		1997-98	urine	52	1 - >16	8	>16	96	
		1997-98	bile	11	0.5 - >16	8	>16	91	
		1997-98	blood	12	8 - >16	8	>16	100	
		Total		77	0.5 - >16	8	>16	92	
	Others	1997-98	all	18	0.25 - 8	1	8	28	
	Total	1997-98		498	0.25 - 32	2	16	61	
Sparfloxacin (Spx)	*E. faecalis*	1997	all	205	0.12 - 64	0.5	1	7	1
		1998	all	198	0.12 - 128	0.5	1	9	
		1997-98	urine	342	0.12 - 64	0.5	1	7	
		1997-98	bile	24	0.12 - 8	0.5	1	8	
		1997-98	blood	32	0.12 - 128	0.5	128	19	
		Total		403	0.12 - 128	0.5	1	8	
	E. faecium	1997	all	38	0.12 - 64	8	64	69	
		1998	all	39	0.12 -128	32	128	76	
		1997-98	urine	52	0.12 -128	8	64	88	
		1997-98	bile	11	0.25 - 64	1	64	46	
		1997-98	blood	12	0.5 - 128	64	128	75	
		Total		77	0.12 - 128	16	128	80	
	Others	1997-98	all	18	0.12 -16	0.5	1	6	
	Total	1997-98		498	0.12 - 128	0.5	16	19	

(To be continued)

Table 7. Antimcrobial susceptibilities of *Enterococcus* species isolated from 1997 to 1998.

Antibiotic	*Enterococcus* spp.	Year	Site	No.	MIC Range	MIC$_{50}$	MIC$_{90}$	% of R'	Bp*
Quinupristin/ Dalfopristin (Str)	*E. faecalis*	1997	all	205	0.5 - 8	4	4	96	1
		1998	all	198	0.12 -16	4	8	93	
		1997-98	urine	342	0.25 - 8	4	8	96	
		1997-98	bile	24	0.5 - 8	4	4	88	
		1997-98	blood	32	0.12 - 8	4	8	84	
		Total		403	0.5 - 16	4	8	95	
	E. faecium	1997	all	38	0.25 - 4	0.5	4	5	
		1998	all	39	0.06 - 8	0.5	2	13	
		1997-98	urine	52	0.06 - 8	0.5	1	10	
		1997-98	bile	11	0.12 - 2	0.5	1	9	
		1997-98	blood	12	0.06 - 0.5	0.12	0.5	0	
		Total		77	0.06 - 8	0.5	1	9	
	Others	1997-98	all	18	0.12 - 4	1	2	44	
	Total	1997-98		498	0.06 - 16	4	8	80	

*BP = breakpoint

R = resistance

those isolated in 1997 (19% and 11%, respectively) ($p < 0.01$). Significantly more *E. faecium* (67%) isolates were resistant to high-level streptomycin (MIC >1024 mg/L) than *E. faecalis* (36%) ($p < 0.01$). The MIC range of streptomycin for *E. faecium* was 32 - >1024 mg/L and the MIC_{50} and MIC_{90} were both >1024 mg/L, while those for *E. faecalis* was 4 ->1024 mg/L, 256 mg/L and >1024 mg/L, respectively. Significantly more *E. faecium* isolates from urine (18%) were resistant than those from bile (9%) ($p < 0.01$).

Significantly more *E. faecium* (92%) isolates were resistant to rifampicin than *E. faecalis* (56%) ($p<0.01$). Consequently, the MIC range of rifampicin for *E. faecalis* was 0.25 - >16 mg/L and MIC_{50} and MIC_{90} were 2 mg/L and 8 mg/L, respectively, while those for *E. faecium* were 0.5 - >16 mg/L, 8 mg/L and >16 mg/L, respectively. More than 50% of *E. faecium* isolates were resistant to trimethoprim and co-trimoxazole (53% and 58%, respectively) while less than 20% of *E. faecalis* isolates were resistant (16% and 15%, respectively).

In contrast, significantly more *E. faecalis* was resistant to chloramphenicol than *E. faecium* (44% and 29%, respectively) ($p < 0.01$). More than 90% of the *E. faecalis* and *E. faecium* isolates were resistant to erythromycin. The MIC range of sparfloxacin (RP 64206) for both *E. faecalis* and *E. faecium* was similar, being from 0.12 mg/L to 128 mg/L. However, the MIC_{50} and MIC_{90} for *E. faecalis* were 0.5 mg/L and 1 mg/L, respectively, while for *E. faecium*, they were 16 mg/L and 128 mg/L, respectively. Significantly more *E. faecium* isolates were resistant to sparfloxacin than *E. faecalis* (66% and 8%, respectively; $p < 0.01$). More *E.*

faecium isolates from urine (88%) were resistant than those from bile (46%) ($p < 0.01$).

The MICs of quinupristin/dalfopristin (RP 59500) for *E. faecalis* ranged from 0.5 mg/L to 16 mg/L and those for *E. faecium* ranged from 0.06 mg/L to 8 mg/L. The MIC_{50} and MIC_{90} for *E. faecalis* were 4 mg/L and 8 mg/L, respectively, and for *E. faecium*, they were 0.5 mg/L and 1 mg/L, respectively. Consequently, significantly more *E. faecalis* isolates were resistant to this combination of drugs than *E. faecium* (17% and 1%, respectively; $p < 0.01$).

Only two (*E. durans* and *E. raffinosus*) of the 18 other enterococcal species were resistant to penicillin or ampicillin. High-level gentamicin resistance was seen in two isolates (both were *E. gallinarum)*. High-level streptomycin was seen in four isolates (*E. gallinarum* (3) and *E. durans* (1)). Only two isolates (*E. gallinarum* and *E. casseliflavus*) were resistant to vancomycin (MIC = 8 mg/L) and none of them was resistant to teicoplanin. More than 80% were resistant to erythromycin, more than 20% to chloramphenicol and rifampicin and 17% to trimethoprim and co-trimoxazole. Less than 10% were resistant to sparfloxacin and none of them were resistant to quinupristin/dalfopristin (RP 59500).

3.3.Detection of vancomycin resistance genes in enterococci by PCR

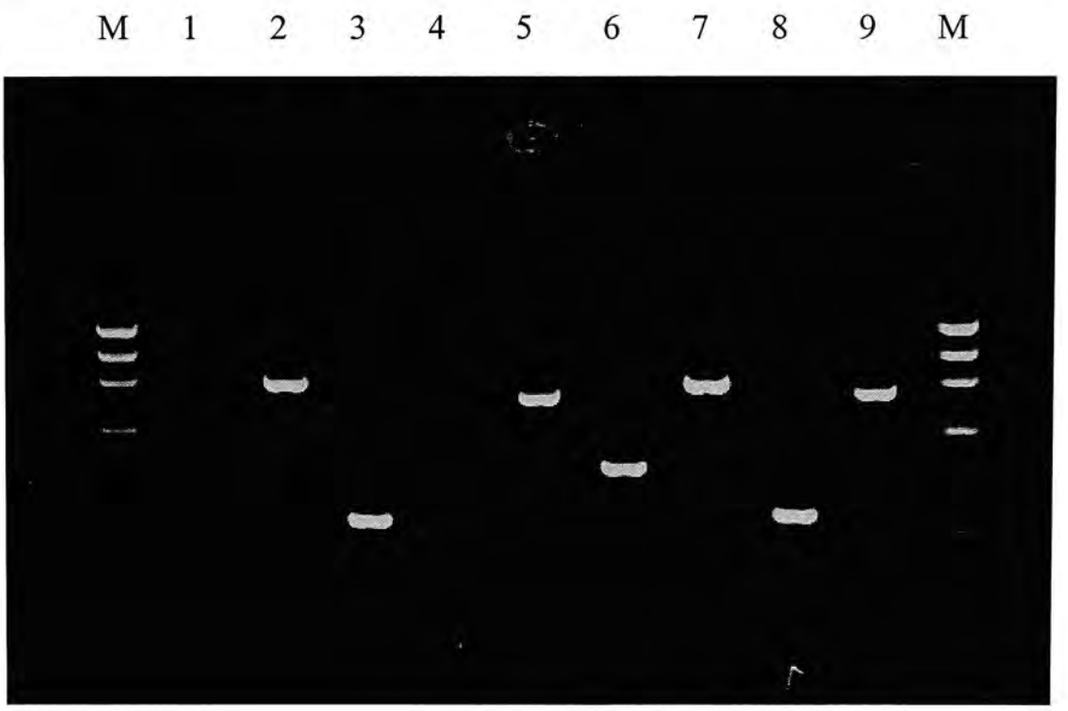
Isolates with vancomycin MICs $\geq$ 4 mg/L were tested for the presence of vancomycin resistance genes by PCR. A total of 47 isolates were tested (*E. faecalis* (35), *E. faecium* (2), *E. gallinarum* (8) and *E. casseliflavus* (2)). They were

subjected to PCR analysis with primers specific to *vanA*, *vanB*, *vanC-1* and *vanC-2/C-3* genes (section 2.6.1). Twelve isolates were found to have *van* genes (Table 8 and Figure 2). The *vanA* gene was detected in one *E. faecium* isolate resistant to both vancomycin (MIC >16 mg/L) and teicoplanin (MIC >16 mg/L). The *vanB* gene was detected in another *E. faecium* isolate that was resistant to vancomycin (MIC >16 mg/L) but sensitive to teicoplanin (MIC = 0.5 mg/L). The *vanC-1* gene was detected in eight *E. gallinarum* isolates and *vanC-2* gene was detected in two *E. casseliflavus* isolates. No amplification products were detected in the remaining 35 isolates (all vancomycin MIC = 4 mg/L).

Table 8. Presence of *vanA*, *vanB*, *vanC-1* and *vanC-2* genes in enterococci with vancomycin MIC ≥ 4 mg/L.

Enterococcus spp.	Van genotype	MIC (mg/L)		No of isolates
		Vancomycin	Teicoplanin	
E. faecium	*vanA*	>16	>16	1
E. faecium	*vanB*	>16	0.5	1
E. gallinarum	*vanC-1*	4 - 8	0.06 - 0.5	8
E. casseliflavus	*vanC-2*	4 - 8	0.06 - 0.25	2
E. faecalis	Nil	4	0.06 - 0.5	35
			Total	47

Figure 2. Agarose gel electrophoresis pattern of amplified products after PCR of enterococci with vancomycin MIC ≥ 4mg/L using primers specific for *van* genes.



Size marker (ϕX174 – *Hae*III digest) in Lane M. Reagent control in Lane 1. Test isolates were contained in lanes 2 –5. Lane 2: isolate with *vanC-1* gene, Lane 3: isolate with *vanB* gene, Lane 4: isolate without *van* gene, Lane 5: isolate with *vanA* gene. Amplified products from control strains in lanes 6 – 9. Lane 6: *E. casseliflavus* (ATCC 25788) with *vanC-2* gene, Lane 7: *E. gallinarum* (AIB39) with *vanC*-1 gene, Lane 8: *E. faecalis* (ATCC 51922) with *vanB* gene, Lane 9: *E. faecium* (AIB40) with *vanA* gene.

3.4. Detection of gene coding for aminoglycoside-modifying enzyme (AAC6'-APH2") by PCR

One hundred and twenty-four isolates (106 of *E. faecalis*, 16 of *E. faecium* and 2 of *E. gallinarum*) with high-level resistance to gentamicin (MIC ≥1024 mg/L) were selected for detection of the presence of *aac(6')-aph(2")* gene. They were subjected to PCR analysis using primers specific to 16S rRNA gene and *aac(6')-aph(2")* gene. All generated the expected amplification product with primers of 16S rRNA gene. One hundred and twenty isolates (102 *E. faecalis*, 16 *E. faecium* and 2 *E. gallinarum*) generated the expected amplification products with primers specific for *aac(6')-aph(2")*. Four *E. faecalis* isolates as well as 20 randomly selected isolates with gentamicin MICs ≤256 mg/L gave no amplification products (Table 9, Figure 3). Thus, these isolates probably harbour genes other than *aac(6')-aph(2")* or did not harbour any aminoglycoside resistance genes.

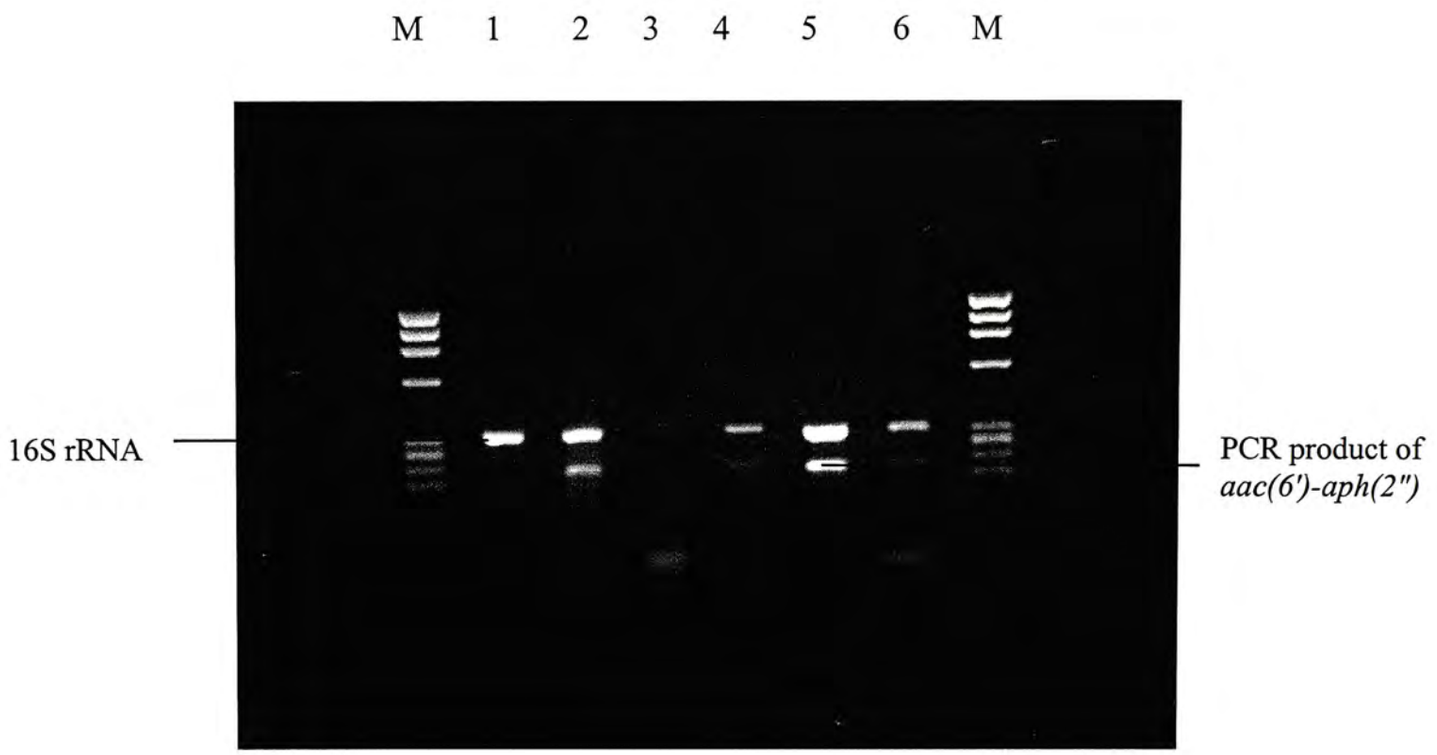
Table 9. Characteristics of enterococci with gentamicin MIC range = 4 - ≥1024 mg/L.

		PCR product using primers for		
Enterococcus spp.	Gentamicin MIC (mg/L)	*16S rRNA*	*aac(6')-aph(2")*	No. of isolates
E. faecalis	>1024	+	+	56
		+	-	3
	1024	+	+	46
		+	-	1
	4 -256	+	-	14
E. faecium	> 1024	+	+	9
	1024	+	+	7
	4 - 16	+	-	4
E. gallinarum	>1024	+	+	2
	8	+	-	1
E. raffinosus	8	+	-	1
			Total	144

+ = PCR product present

- = No PCR product

Figure 3. Agarose gel electrophoresis pattern of amplified products obtained by PCR using primers specific for 16S rRNA and *aac(6')-aph(2")* genes.



Size marker (ϕX174 – *Hae*III) digest in Lane M. Amplified products from control strains in lane 1: *S. aureus* (ATCC 29213) and lane 2: *S. aureus* (77040101). Reagent control in Lane 3. Tested isolated were contained in lanes 4 – 6. Lane 4 - 6: presence of *aac(6')-aph(2")* gene.

3.5. Detection of β-lactamase in enterococci

Seventy-six isolates (72 of *E. faecium*, three of *E. faecalis* and one of *E. durans*) with ampicillin MIC >8 mg/L were tested for the production of β-lactamase using cefinase disc. β-Lactamase was not detected in all the isolates tested.

3.6. Resistance pattern of enterococci

Isolates were regarded as resistant to an antibiotic if the MIC was greater than the concentration of that antibiotic for susceptible strains according to the recommendation of the NCCLS (1999). Only two isolates were susceptible to all the antibiotics tested while the rest were resistant to one to 10 antibiotics in a total of 88 patterns (Table 10). About 40% of the isolates were resistant to one to three antibiotics and 17% were resistant to more than seven antibiotics. There were 20 isolates that were resistant to nine antibiotics with the resistance pattern A P G St E Tm Sxt C Rif Spx being found in 19 (18 *E. faecium*, 1 *E. faecalis*) of them. The most common resistance pattern was E Rif Str being found in 14% of isolates (67 of *E. faecalis* and one other enterococcal species), followed by E Str (12%, 56 of *E. faecalis* and 2 other enterococcal species), E C Rif Str (6%, 31 of *E. faecalis* and one other enterococcal species) and St E C Rif Str (5%, 27 of *E. faecalis*). The other resistance patterns (except A P G St E Tm Sxt C Rif Spx) were each found in 1 – 17 (0.2 – 3%) isolates.

Table 10. Antibiotic resistance phenotypes of the 498 enterococcal isolates.

No. of antibiotic resistance	Resistance pattern	No. of strains			Total (%)
		E. faecalis	*E. faecium*	Others	
0	-	-	-	2	2 (0.4)
1	E	5	1	4	10
	Str	3	-	-	3
	Total				13 (2.6)
2	E Str	56	-	2	58
	E Rif	5	2	1	8
	Rif Str	2	1	-	3
	V E	-	-	1	1
	Total				70 (14)
3	E Rif Str	67	-	1	68
	E C Str	17	-	-	17
	St E Str	13	-	-	13
	G E Str	8	-	-	8
	C Rif Str	2	-	-	2
	P E Str	-	-	1	1
	E C Rif	1	-	-	1
	E Tm Sxt	1	-	-	1
	V E Rif	-	-	1	1
	Total				112 (22)
4	E C Rif Str	31	-	1	32
	St E Rif Str	17	-	-	17
	St E C Str	11	-	-	11
	G E Rif Str	8	-	-	8
	E Tm Sxt Str	6	-	1	7
	G E C Str	7	-	-	7
	G St E Str	5	-	-	5
	St E C Rif	3	-	-	3
	St E Spx Str	3	-	-	3
	E Rif Spx Str	2	-	-	2
	A P E Rif	-	1	-	1
	E Tm Sxt C	1	-	-	1
	G E Spx Str	1	-	-	1
	G E Tm Sxt	1	-	-	1
	G Rif Spx Str	1	-	-	1
	Total				100 (20)

- : Nil

(To be continued)

Table 10. Antibiotic resistance phenotypes of the 498 enterococcal isolates.

No. of antibiotic resistance	Resistance pattern	No. of strains			Total
		E. faecalis	*E. faecium*	Others	
5	St E C Rif Str	27	-	-	27
	G E C Rif Str	10	-	-	10
	G E Tm Sxt Str	5	-	-	5
	G St E Rif Str	4	-	-	4
	E Tm Sxt C Str	3	-	-	3
	G St E C Str	3	-	-	3
	A P E Rif Spx	-	2	-	2
	G St E Spx Str	2	-	-	2
	E Tm Sxt Rif Str	2	-	-	2
	A P C Rif Spx	-	1	-	1
	A P St E C	-	-	1	1
	G E Rif Spx Str	1	-	-	1
	St E C Spx Str	1	-	-	1
	St E Tm Sxt Rif	1	-	-	1
	St E Tm Sxt Spx	1	-	-	1
	St E Tm Sxt Str	1	-	-	1
	St E Rif Spx Str	1	-	-	1
	Total				66 (13)
6	G St E C Rif Str	16	-	-	16
	A P St E Rif Spx	-	12	-	12
	St E Tm Sxt C Str	5	-	-	5
	G St E C Spx Str	4	-	-	4
	A P G E Rif Spx	-	3	-	3
	G E Tm Sxt Rif Str	3	-	-	3
	G E Tm Sxt C Str	2	-	-	2
	A P V E Tm Sxt	-	1	-	1
	E Tm Sxt C Spx Str	1	-	-	1
	E Tm Sxt C Spx Str	1	-	-	1
	G E Tm Sxt Spx Str	1	-	-	1
	G St E Tm Sxt Str	1	-	-	1
	St E Tm Sxt Rif Str	1	-	-	1
	Total				51 (10)

-: Nil

(To be continued)

Table 10. Antibiotic resistance phenotypes of the 498 enterococcal isolates.

No. of antibiotic resistance	Resistance pattern	No. of strains			Total
		E. faecalis	*E. faecium*	Others	
7	G St E Tm Sxt C Str	6	-	1	7
	A P E Tm Sxt Rif Spx	-	4	-	4
	A P St E Tm Sxt Rif	-	4	-	4
	G E Tm Sxt C Rif Str	3	-	-	3
	G St E C Rif Spx Str	3	-	-	3
	St E Tm Sxt C Rif Str	3	-	-	3
	A P G St E C Rif	-	1	-	1
	A P G E Tm Sxt Rif	-	1	-	1
	A P G E Rif Spx Str	-	1	-	1
	A P St E Rif Spx Str	-	1	-	1
	A P St Tm Sxt Rif Spx	-	1	-	1
	G E Tm Sxt C Spx Str	1	-	-	1
	G St E Tm Sxt Spx Str	1	-	-	1
	P G St E C Spx Str	1	-	-	1
	St E Tm Sxt C Spx Str	1	-	-	1
	Total				33 (6.6)
8	A P St E Tm Sxt Rif Spx	-	9	-	9
	G St E Tm Sxt C Spx Str	5	-	1	6
	A P G E Tm Sxt Rif Spx	-	4	-	4
	G St E Tm Sxt C Rif Str	3	-	-	3
	A P St E Tm Sxt C Rif	-	2	-	2
	A P G E C Rif Spx Str	-	1	-	1
	A P G E Tm Sxt C Rif	-	1	-	1
	A P G St E Tm Sxt Spx	-	1	-	1
	A P V Tei G E Rif Spx	-	1	-	1
	Total				28 (5.6)
9	A P St E Tm Sxt C Rif Spx	1	18	-	19
	A P G St E Tm Sxt Rif Spx	-	1	-	1
	Total				20 (4)
10	A P St E Tm Sxt C Rif Spx Str	1	1	-	2
	A P G St E Tm Sxt C Rif Spx	-	1	-	1
	Total				3 (0.6)
				Total	498 (100)

- : Nil

3.7. Multi-resistant enterococci investigated by pulsed-field gel electrophoresis

Isolates that satisfied the following criteria were selected for molecular typing by pulsed-field gel electrophoresis:

I. Resistant to the breakpoint concentration of an antibiotic for resistant strains recommended by the NCCLS (i.e. penicillin [P] >8 mg/L, ampicillin [A] >8 mg/L, vancomycin [V] ≥8 mg/L, teicoplanin [Tei] ≥8 mg/L, gentamicin [G] >512 mg/L, streptomycin [St] >1024 mg/L, erythromycin [E] ≥4 mg/L, trimethoprim [Tm] >8 mg/L, cotrimoxazole [Sxt] >32 mg/L, chloramphenicol [C] ≥16 mg/L, rifampicin [Rif] ≥2 mg/L, sparfloxacin [Spx] ≥2 mg/L and quinupristin/dalfopristin (RP 59500) [Str] ≥2 mg/L);

II. Simultaneously resistant to eight or more antibiotics; and

III. With the most common resistance phenotypes.

According to the above criteria, 50 isolates (42 *E. faecium* isolates and 8 *E. faecalis* isolates) were selected. Based on their resistance phenotypes, the *E. faecium* isolates could be divided into four groups according to their resistance to ampicillin, gentamicin and/or streptomycin (high concentration) in addition to various other antibiotics (Table 11). Group I consisted of three isolates with ampicillin resistance and high-level gentamicin and streptomycin resistance, group II consisted of six isolates with ampicillin and high-level gentamicin resistance, group III consisted of 32 isolates with ampicillin and high-level streptomycin resistance and group IV consisted of two isolates with ampicillin resistance.

Table 11. Distribution of *E. faecium* by resistance patterns.

Group	Resistance pattern	No. of isolates
I a	**A** P **G** **St** E Tm Sxt Rif Spx	2
b	**A** P **G** **St** E Tm Sxt Spx	1
II a	**A** P **G** E Tm Sxt Rif Spx	3
b	**A** P **G** E Tm Sxt C Rif	1
c	**A** P **G** E C Rif Spx Str	1
d	**A** P V Tei **G** E Rif Spx	1
III a	**A** P **St** E Tm Sxt Rif Spx	19
b	**A** P **St** E Tm Sxt Rif	8
c	**A** P **St** E Tm Sxt C Rif Spx	3
d	**A** P **St** E Tm Sxt C Rif	1
IV	**A** P E Tm Sxt Rif Spx	2
	Total	42

By PFGE, the 42 *E. faecium* isolates could be typed into a total of 37 PFGE types (types 1 – 37). All except five types consisted of one isolate each. These five types were 1, 4, 7, 19 and 21 and each consisted of two isolates. Twenty-one types were of ≥90% similarity and could be grouped into eight groups (groups A to H) (Figure. 4). Group A consisted of four types, groups C, E and F consisted of three types each and groups B, D, G and H consisted of two types each.

Table 12 shows the relationship between resistance patterns and PFGE types of *E. faecium*. Resistance pattern III a was the most common, being exhibited by 45% (19) of isolates. Among these isolates, 14 could be grouped into five different PFGE group (A, B, C, E, H) while the remaining isolates were of <90% similarity. The next most common resistance pattern was III b exhibited by 19% (8) of isolates. Three isolates belonged to the same PFGE group (F), one belonged to group C and the rest belonged to four types of <90% similarity. Isolates of the same resistance pattern could be of different PFGE types or groups while strains with the same PFGE types or groups could be resistant to different spectra of antibiotics.

The eight *E. faecalis* isolates selected for PFGE could be divided into three groups (groups I - III) according to their resistance pattern. All were resistant to high-level gentamicin and streptomycin except one that was resistant to streptomycin and ampicillin but not gentamicin (Table 13). Group I (G St E Tm Sxt C Spx Str) consisted of four isolates and group II (G St E Tm Sxt C Rif Str)

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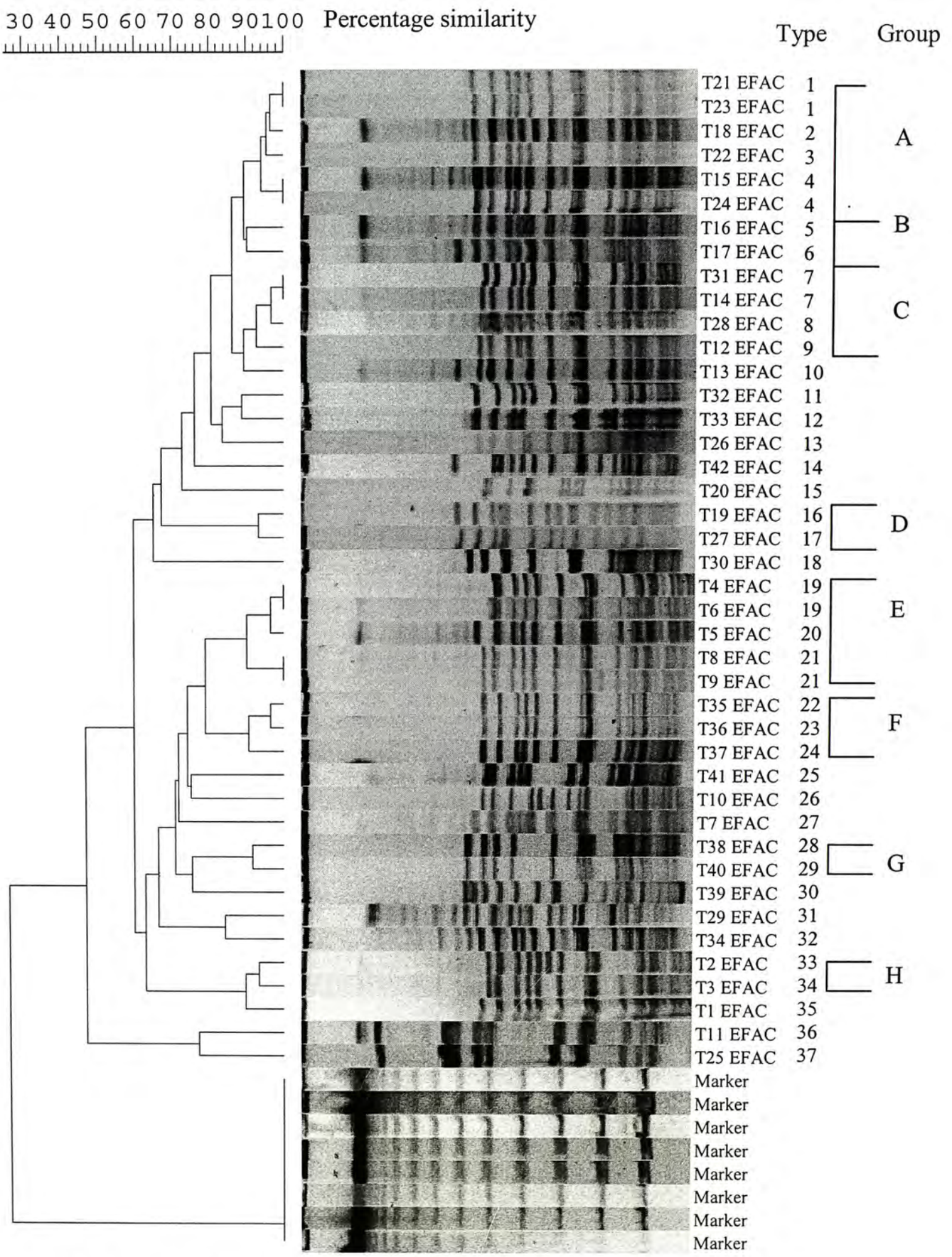
consisted of three isolates. Group III (A P St E Tm Sxt Rif Spx) consisted of only one isolate. These *E. faecalis* isolates could be typed into a total of seven PFGE types and only two (types 4 and 5) were of >90% similarity (Figure 5). Two isolates were of the same PFGE type (type 1). Table 14 shows the relationship between resistance patterns and PFGE types of *E. faecalis*. Isolates of the same resistance patterns belonged to different PFGE types, a finding similar to that of *E. faecium*.

Figure 4. Dendrogram showing relationships between the 42 multi-resistant *E. faecium* based on their PFGE patterns of *Sma*I-digested genomic DNA.



Marker : λ-DNA PFGE marker (kb)

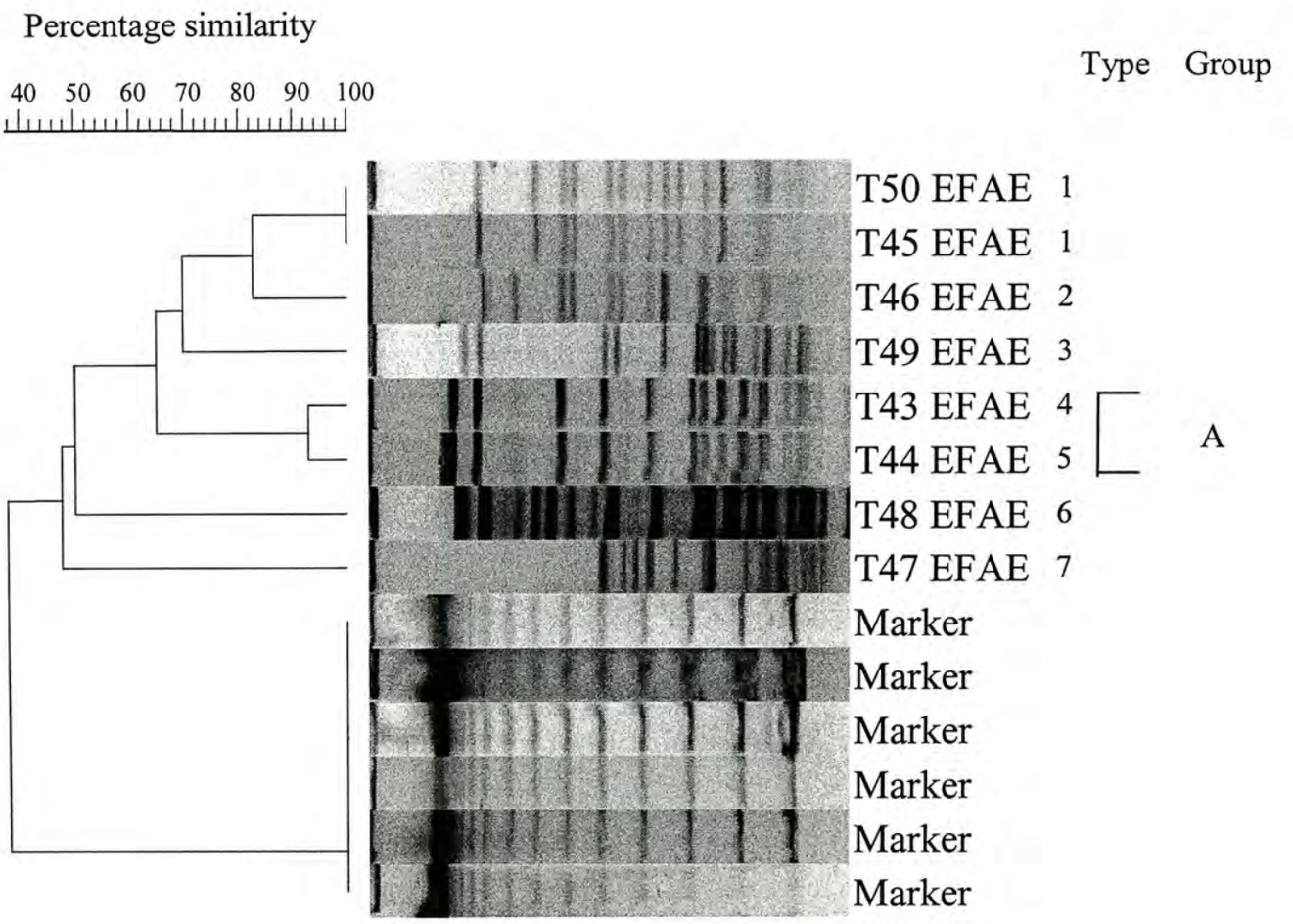
Table 12. Correlation of resistance patterns and PFGE types of *E. faecium*.

PFGE		Resistance pattern											
Group	Type	I a	I b	II a	II b	II c	II d	III a	III b	III c	III d	IV	Total
A	1	1								1			2
	2							1					1
	3									1			1
	4							1				1	2
B	5							1					1
	6							1					1
C	7							2					2
	8								1				1
	9							1					1
D	16					1							1
	17	1											1
E	19							2					2
	20							1					1
	21							2					2
F	22								1				1
	23								1				1
	24								1				1
G	28			1									1
	29			1									1
H	33							1					1
	34							1					1
-	10							1					1
	11								1				1
	12								1				1
	13		1										1
	14						1						1
	15				1								1
	18					1			1				1
	25									1			1
	26							1					1
	27							1					1
	30			1									1
	31								1				1
	32										1		1
	35							1					1
	36							1					1
	37											1	1
	Total	2	1	3	1	2	1	19	8	3	1	2	42

Table 13. Distribution of multi-resistant *E. faecalis* by resistance patterns.

Group	Resistant phenotype	No. of strains
I	G St E Tm Sxt C Spx Str	1
	G St E Tm Sxt C Spx Str	2
	G St E Tm Sxt C Spx Str	1
II	G St E Tm Sxt C Rif Str	1
	G St E Tm Sxt C Rif Str	1
	G St E Tm Sxt C Rif Str	1
III	A P St E Tm Sxt Rif Spx	1
	Total	8

Figure 5. Dendrogram showing relationships between the eight multi-resistant *E. faecalis* isolates based on their PFGE patterns of *Sma*I-digested genomic DNA.



Marker: λ DNA-PFGE marker

Table 14. Correlation of resistance pattern and PFGE types of *E. faecalis*.

PFGE		Resistance pattern			
Group	Type	I	II	III	Total
A	4		1		1
	5		1		1
-	1	1			1
	2	2			2
	3	1			1
	6		1		1
	7			1	1
	Total	4	3	1	8

4. Discussion

During the past twenty years, enterococci have emerged as important pathogens because of their association with nosocomial infections. They are increasingly being isolated from hospitalized patients with urinary tract infections, bacteraemia and wound infections (Murray, 1990; Schaberg, Culver & Gaynes, 1991). Besides being intrinsically resistant to various antibiotics, they also acquired resistance to many antibiotics. With the acquisition of glycopeptide resistance genes from other bacterial species, vancomycin-resistant enterococci (VRE) have developed and become widespread in many places. Workers in Europe showed that glycopeptide-resistant enterococci have been isolated from both animal and human faeces (Gordts *et al*, 1995; Welton *et al*, 1998; Phillips, 1999). In contrast, such strains were mainly isolated from hospitalized patients in the United States (Murray, 1997). This was probably due to the widespread use of avoparcin, a glycopeptide used as a growth promotor, in animals in Europe while the drug was banned for animal use in 1997 in the United States. It could be speculated that the prevalence of VRE infection in the United States was due to extensive use of glycopeptides in humans while in Europe, it was due to the increased use of this group of antibiotics in both humans and animals (French, 1998; Phillips, 1999). Thus, the only strategy against these organisms is to prevent their spread by strict infection control measures (Spera & Farber, 1992; Murray, 1995; Heath, Blackmore & Gordon, 1996; Leclercq, 1996).

The situation of enterococcal infections and their antibiotic resistance in Hong Kong is largely unknown. We therefore set out to determine the distribution

of enterococci and their antibiotic susceptibilities in a large general hospital in Hong Kong. It is hoped that results of this study would elucidate the types and levels of antibiotic resistance of enterococcal infections in Hong Kong. Clinicians could therefore be guided for the best choice of antimicrobial agents in treating their patients with enterococcal infections. Indeed, we have revealed that antibiotic resistance among our enterococcal strains was high. This would be discussed further in the section on antibiotic susceptibilities of enterococci.

Enterococci isolated from urine, bile, blood, body fluids and CSF were selected for the study. This is because urinary tract infection is the most common infection caused by enterococci (Murray, 1990) while serious infections such as endocarditis, septicaemia, meningitis, etc can also be caused by enterococci. Since more than a thousand enterococcal isolates were isolated from urine and bile every year, we could only manage to test a limited but reasonable number of isolates from these specimens. By testing isolates collected during the same period (April to June) in two consecutive years, we might be able to detect changes, if any, in the prevalence of antibiotic resistance. In contrast, many fewer isolates were from blood, body fluid and CSF so that isolates from these specimens collected throughout a year were studied (April, 1997 to March, 1998). Isolates from wound specimens were excluded from the study because their clinical significance is questionable (Low, Willey & McGeer, 1995; Facklam & Sahm, 1995).

This study showed that the majority of enterococci were isolated from urine (80%) even though organisms isolated from this type of specimen were collected

only during a six-month study period while organisms from blood which made up 10% of the isolates studied were collected over 12 months. Organisms from bile, body fluids and CSF made up the remaining 10% of the isolates collected. Thus, urinary tract infection was the most common infection caused by enterococci. Besides, more isolates were collected from urine and bile in 1997 than in 1998. Whether this was due to a decrease in the number of enterococcal infections or an overall decrease in the number of infections in 1998 had to be evaluated by analyzing the total number of all infections in this year.

The distribution of enterococci in different specimens was similar to that of other studies. In their study in the United States, Gordon *et al* (1992) found that the majority of isolates are isolated from the urinary tract (57%) followed by non-surgical wound (13.3%), blood (10.5%), surgical wound (8.8%) and miscellaneous sites (10.4%). McNamara and colleagues (1995) also found that the majority of their strains are isolated from the urinary tract (66%) with those from wound and soft tissue (23%) being next in prevalence and only 3% are isolated from blood.

More than 80% of the enterococci studied were *E. faecalis* (81%) while *E. faecium* made up only 15%. The other *Enterococcus* species were rarely isolated (4%). This distribution of enterococcal species was also similar to that found in other studies. In the series of Gordon *et al* (1992) and Vandamme *et al* (1996), the respective figures are 90%, 8-9% and 2%. In contrast, in Greece during 1993-1994, Douboyas & Tsakris (1995) found that of their 224 isolates, only 48% were *E. faecalis* while *E. faecium* made up 29%. Although almost 70% of the isolates

reported by Perlada and colleagues (1997) were identified as *E. faecalis* (69%), *E. faecium* constituted (29%) of their collection of enterococci. The distribution of species thus varies in different countries. This further supports the fact that we should study the distribution of enterococcal species to reflect the local situation.

Conventional biochemical method is the "gold standard" used in the identification of enterococci. Commercially available biochemical kits such as API 20 Strep and API 32 Strep are now widely used despite of their high price because they are easy and simple to use. Recently, molecular methods such as PCR that is more rapid have been used in the identification of enterococci. Four clinically-important enterococcal species *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus* can be definitively identified using specific primers in PCRs (Dutka-Malen, Evers & Courvalin, 1995; Free & Sahm, 1996). Since our laboratory mainly used API 20 Strep to identify enterococci to species level, we investigated the possibility of using PCR as an alternative method. Similar to findings of other workers (Nachamkin, Lynch & Dalton, 1982; Jorgensen, Crawford & Alexander, 1983; Facklam, Rhoden & Smith, 1984; Facklam *et al,* 1985; You & Facklam, 1986; Ruoff *et al*, 1990; Teixeria *et al*, 1995; Donabedian *et al*, 1995), we found that enterococcal species other than *E. faecalis* could not be accurately identified by API 20 Strep: only three out of the 243 isolates (1%) of *E. faecalis* but seven of 52 isolates (13%) of *E. faecium* and 12 (86%) of 14 isolates of other enterococcal species were misidentified. Conventional biochemical tests had to be carried out to give a definitive identification of these organisms. Recently, the use of API has been evaluated against BBL crystal systems for the identification of VRE. API 20

Strep could only correctly identify 57% of *E. faecium* while the Crystal Gram Positive and Rapid Gram Positive kits could correctly identify 93% and 96%, respectively of the strains (Hamilton-Miller & Shah, 1999). Although BBL crystal systems appeared to be superior to API 20 Strep in identifying enterococci, they are not available in Hong Kong.

Both PCR and conventional biochemical tests gave the same identification results for 63 of the 69 non-*E. faecalis* isolates identified by API 20 Strep. These included 52 *E. faecium* isolates, five *E. gallinarum* isolates, three isolates of each *E. casseliflavus* and *E. faecium*. Six isolates failed to give any amplification product since only primers specific for the gene coding for D-alanine:D-alanine ligases in *E. faecalis* or *E. faecium* ($ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$) and *vanC-1* or *vanC-2/C-3* genes of *E. gallinarum* or *E. casseliflavus/E. flavescens,* respectively, were used. Isolates that were not these enterococcal species had to be identified by supplementary tests such as hydrolysis of arginine, fermentation of mannitol, sorbitol, arabinose and raffinose, motility and pigment production (Facklam & Collin, 1989). Thus, PCR could only correctly identify *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus* while for other species, specific primers had to be designed (Dutka-Malen, Evers & Courvalin, 1995; Arthur, Reynolds & Courvalin, 1996; Free & Sahm, 1996; Clark *et al*, 1998).

The *vanC-1* and *vanC-2/C-3* genes are present intrinsically in *E. gallinarum* and *E. casseliflavus/E. flavescens,* respectively, that is, they are chromosomally-mediated and are not easily transferred to other species (Toye *et al*, 1997; Clark *et*

al, 1998). However, the possibility of transfer of these genes by transformation to other bacterial organism cannot be excluded. Therefore, supplementary tests (motility and pigment formation) should be performed to confirm the identity of *E. gallinarum* and *E. casseliflavus/E. flavescens* when they were identified as such by PCR.

Although PCR could correctly identify clinically important enterococci, namely *E. faecalis* and *E. faecium*, this method might not be suitable for use in a routine Microbiology laboratory. Firstly, special techniques and equipment were required. Secondly, it would not be cost-effective if too few isolates were tested at one time. However, it could be used under certain circumstances especially when presence of vancomycin-resistant enterococci (VRE), high-level gentamicin-resistant or β-lactamase-producing enterococci were suspected. As PCR could provide a definitive identification within a few hours, it would be very useful for infection control purposes as health care personnel could be alerted to take prompt action to prevent the spread of the organism.

E. faecium was found to be more antibiotic resistant than *E. faecalis* as noted in other localities (McNamara, King & Symth; 1995; Perlada, Smulian & Cushion, 1997; Huycke, Sahm & Gilmore, 1998). They are also more resistant to penicillin, ampicillin, streptomycin, trimethoprim, rifampicin and ciprofloxacin than other enterococcal species (Gordon *et al*, 1992; Vandamme *et al*, 1996; Tsakris, Pournarsa & Douboyas, 1997; Reinert *et al*, 1999; Andrews *et al*, 2000). In this study, a large proportion of *E. faecium* isolates was resistant to penicillin

and ampicillin (65%), streptomycin (45%), trimethoprim (34%), rifampicin (60%) and ciprofloxacin (10%). In contrast, the corresponding figures for *E. faecalis* were 5%, 20%, 15%, 30% and 5%.

Although VRE remain a major problem in hospitals world-wide (Kaplan, Gilligan & Facklam, 1988; Murray, 1995; Shay *et al*, 1995; Murray, 1997; Noskin, 1997), we only detected 12 VRE isolates from the Prince of Wales Hospital in 1998. This was the first batch of VRE found in Hong Kong. Two of these were *E. faecium,* one phenotypically VanA (resistant to both vancomycin and teicoplanin) and the other phenotypically VanB (resistant to vancomycin but susceptible to teicoplanin). The other 10 isolates were *E. gallinarum* (8) and *E. casseliflavus* (2), exhibiting low-level vancomycin resistance (MIC of vancomycin 4 – 8 mg/L) of VanC phenotype. Only a few other places such as in Ireland, Greece and Germany where a low prevalence of VRE was reported. This may be due to the limited use of vancomycin, avoparcin and other broad-spectrum such as cephalosoprins, and stringent infection control procedures (McNamara, King & Symth, 1995; Vandamme *et al*, 1996; Tsakris, Pournaras & Douboyas, 1997; French, 1998; Reinert *et al*, 1999). The very small number of VRE isolated in our hospital might probably be due to the fact that our hospital had an efficient infection control team that was responsible for preventing the spread of infections within the hospital. In addition, the use of avoparcin as a growth promoter for food animals might not be widespread in Hong Kong.

A similar proportion of *E. faecalis* and *E. faecium* were resistant to high-levels of gentamicin (26% and 21%, respectively) but the percentage of gentamicin-resistance enterococci isolated in 1998 was higher than in 1997, namely *E. faecalis* (34% in 1998 vs 19% in 1997) and *E. faecium* (32% in 1998 and 11% in 1997). Significantly more *E. faecium* were resistant to streptomycin than *E. faecalis* (71% versus 43%). A much smaller proportion of the other enterococcal species were resistant to high-level gentamicin and streptomycin (11% and 22%, respectively). These figures were similar to those reported in Irish, Greek and German hospitals (McNamara, King & Smyth, 1995; Tsakris, Pournaras & Douboyas, 1997; Reinert *et al*, 1999).

Most of the enterococci isolates were resistant to nalidixic acid and the fluoroquinolones (Greenwood, 1989). However, the more recently developed fluoroquinolones exhibited a moderate degree of activity. Sparfloxacin belonged to the third generation fluoroquinolones which has been shown to have a better activity against gram-positive organisms (Qadri, 1992). Since NCCLS did not recommend a breakpoint concentration for sparfloxacin, we chose 1 mg/L, a value which is the same as that for ciprofloxacin. Although 19% of enterococci were resistant to sparfloxacin, most of the resistant strains were *E. faecium,* and more than 90% of *E. faecalis* and other enterococcal species were susceptible. In the study of Qadri (1992), sparfloxacin showed excellent activity against enterococci with 92% of all the 59 isolates being susceptible to sparfloxacin at 4 mg/L, while only 22% and 27% are susceptible to norfloxacin and ciprofloxacin, respectively.

Quinupristin/dalfopristin (RP 59500) is the combination of two new semisynthetic water-soluble streptogramins with good activity against enterococci other than *E. faecalis* (Cormican & Jones, 1996; Finch, 1996; Witte, Wirth & Klare, 1999). It is also active against most glycopeptide resistant strains of *E. faecium* (Collins *et al*, 1993; Eliopaulos *et al*, 1998). The two isolates of vancomycin-resistant *E. faecium* with Van A and Van B genotype were susceptible to quinupristin/dalfopristin (MICs = 0.25 mg/L and 1 mg/L, respectively). However, 9% of all *E. faecium* isolates tested were resistant to quinupristin/dalfopristin (MIC_{90} = 1 mg/L). It is surprisingly to find so much resistance to quinupristin/dalfopristin in this study as the drug was not yet available in Hong Kong or even anywhere in the world. Although a close relative, pristinamycin, has been available in France for many years, this is still not available in Hong Kong. Reinert *et al* (1999) showed that their *E. faecium* isolates which habour *vanA* gene are susceptible to quinupristin/dalfopristin. The fact that 95% of our *E. faecalis* isolates and 44% of other enterococcal species were resistant to quinupristin/dalfopristin suggested that the indication of this drug was mainly for *E. faecium*.

In general, the percentage of enterococci resistant to various antibiotics in this study was considerably higher as compared to that in other countries. Ninety-five percent of our *E. faecium* isolates were resistant to ampicillin while other studies reported only 50% - 78% (Huygens & Lewis, 1995; Struwig, Botha & Chalkley, 1998; Andrews *et al*, 2000). More than 20% of our enterococci exhibited high-level resistance to gentamicin, while in other places the figure was 14% -

20% (Silverman *et al*, 1998; Reinhert *et al*, 1999, Schouten *et al*, 1999). Whether the large proportion of resistant strains was a result of the extensive use of antibiotics that exerted selective pressures to facilitate resistance gene transfer (Woodford *et al*, 1992 & Simjee & Gill, 1997) had to be elucidated by reviewing the amount of antibiotic consumption supplied by the hospital pharmacy.

Although enterococci are intrinsically resistant to aminoglycosides, the treatment of serious enterococcal infections is the synergistic combination of a β-lactam or glycopeptide with an aminoglycoside (Murray, 1990). However, this synergy would be lost if high-level aminoglycoside resistance developed and such therapy would not be effective. Our study showed that an average of 15% of the enterococcal isolates were resistant to ampicillin and 25% were resistant to high concentrations of gentamicin. Thus, there would be a 1 in 4 chance of treatment failure if the standard regimen for treating serious enterococcal infections was used. Resistance to other drugs such as cotrimoxazole, erythromycin or chloramphenicol was also high so that they would also not be useful as therapeutic agents for enterococcal infection. However, vancomycin and teicoplanin remained effective as only a very small proportion of enterococci was resistant to them.

We used PCR to detect the presence of vancomycin resistance genes in strains with vancomycin MICs ≥4 mg/L. The primers chosen were specific for *vanA*, *vanB*, *vanC-1* and *vanC-2/C-3* (Dutka-Malen, Evers & Courvalin, 1995; Free & Sahm, 1996; Clark *et al*, 1998). There were only one isolate each of VanA and VanB resistance and both were *E. faecium*, while there were eight isolates of

E. gallinarum with VanC-1 resistance and two isolates of *E. casseliflavus* withVanC-2 resistance. The clinical significance of *vanA* and *vanB* genes has been well-documented (Arthur & Courvalin, 1993; Quintiliani, Evers & Courvalin, 1993; Low, Willey & McGeer, 1995; Arthur, Reynolds & Courvalin, 1996; Leclercq, 1997; Murray, 1997; French, 1998). However, whether strains with *vanC-1* and *vanC-2/C-3* are clinically significant is still questionable since only low-level vancomycin resistance is conferred and special infection control procedures are usually not required (Low, Willey & McGeer, 1995; Toye *et al*, 1997; Clark *et al*, 1998). However, high-level resistance to glycopeptides has also been reported in *E. gallinarum* and *E. casseliflavus* (Dutka-Malen *et al*, 1994; Yoshikazu *et al*, 1996; Coombs *et al*, 1999). Thus, detection of *vanA*, *vanB* and *vanC* is recommended whenever vancomycin-resistant enterococci were isolated from clinical specimens (Coombs *et al*, 1999).

High-level gentamicin resistance in enterococci is usually due to acquisition of genes coding for aminoglycoside-modifying enzymes (AMEs) that inactivated the activity of gentamicin. The bi-functional enzyme 6'-N-acetyltransferase/2" phosphotransferase (AAC6-APH2") which mediates high-level resistance to gentamicin and to all clinically useful aminoglycosides (gentamicin, tobramycin, amikacin and netimicin) except streptomycin. It is the most common AME detected in enterococci (Murray, 1990; Leclercq, 1997; Moellering, 1997). This enzyme is also commonly produced by staphylococci and is thought to be the origin of the enzyme in enterococci (Leclerq *et al*, 1992). We performed PCR using primers specific for *aacA/aphD* that codes for AAC6'-

APH2" (van der Klundert & Vlegenthart, 1993) on all our isolates with gentamicin MICs ≥1024 mg/L. Only four of the 124 isolates tested did not habour the gene. This showed that most of our gentamicin-resistant enterococcal isolates were due to the production of AAC6'-APH2" enzyme. As van den Braak *et al* (1999) showed that enterococci can produce more than one AME, we had to confirm whether our isolates produced AAC6'-APH2" enzyme in addition to other enzymes by PCR using primers specific for these other enzymes. The mechanism of high-level gentamicin resistance in the four strains with no amplification product after PCR needs to be further investigated. The resistance might be due to other AMEs found in enterococci such as 6-nucleotidyltransferase (ANT6), 4'-nucleotidyltransferase (ANT4'), 3'-phosphotransferase (APH3') or APH(2") (Murray, 1990; Leclercq *et al*, 1992; Tsai *et al*, 1998), novel undescribed enzymes or resistance mechanisms other than enzyme hydrolysis.

None of the 76 isolates of ampicillin-resistant enterococci were found to produce β-lactamase. This is not surprising as β-lactamase has only been detected rarely in enterococci (Greenwood, 1989; Gray and Pedler, 1992). Resistance to β-lactams is usually due to the decreased affinity of the drug to penicillin binding proteins. Thus, further work has to be carried out to determine whether the mechanism of resistance to the β-lactam drugs was due the alteration or decreased affinity of PBP5 (Fontanna *et al*, 1994; Rybkine *et al*, 1997).

By using the breakpoint criteria for susceptible strains (NCCLS, 1999), only two (0.4%) out of the 498 isolates tested were susceptible to all the antibiotics

tested while 60% were resistant to more than three antibiotics tested. In order to determine there was any clonal relationship between multi-resistant enterococci, PFGE was used to fingerprint 42 *E. faecium* and eight *E. faecalis* isolates that were resistant to seven to nine antibiotics. PFGE types of ≥85% similarity were grouped within one group. *E. faecium* isolates of the same resistance phenotypes were found to belong to different PFGE types or PFGE groups. Similarly, although the *E. faecalis* isolates tested were of similar resistance patterns, they belonged to different PFGE types or groups. Thus, there was no correlation between antimicrobial resistances and DNA fingerprints obtained by PFGE. Thus, these enterococcal infections were most probably sporadic cases and not due to spread of a few related strains.

In summary, the majority of the enterococci isolated in the present study were *E. faecalis* (81%) followed by *E. faecium* (15%) and other enterococci (4%). Most of the isolates were from urine. *E. faecium* was generally more resistant to antibiotics than *E. faecalis*. Fifteen percent of the enterococci studied were resistant to ampicillin. High-level gentamicin resistance was detected in 18% and was due mainly to the production of AAC6'-APH2" enzyme. Two *E. faecium* isolates, one of VanA and the other of VanB phenotype were the only clinically significant VRE detected. Multi-resistant enterococci were shown to be heterogeneous by PFGE.

5. Future Work

This study has opened ample opportunities for various research areas to be carried out in future.

1. Continuous surveillance of antimicrobial susceptibilities of enterococci should be performed so as to monitor the development of VRE in the hospital and in the community. Prompt identification of VRE in the hospital could ensure that strict infection control measures could be instituted to prevent the spread of the organism. By screening for VRE carriers in the community, the prevalence of VRE could be determined and the possibility of development of VRE infections could be predicted.

2. At present, primers specific for genes coding for D-alanine: D-alanine (D-Ala: D-Ala) ligases (ddl) and D-ala-D-serine ligases were available for the identification of *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus/E. flavescens* only. Primers specific for enterococcal species other than these could be designed so that PCR could be applied for the identification for more enterococcal species.

3. Only 2% of the isolates with elevated vancomycin MICs were shown to harbour *vanA*, *vanB* or *vanC* genes. Presence of *vanD*, *vanE* or new *van* genes could be investigated by using *van* degenerated primers. The amplified products could be cloned and subsequently sequenced. If a new gene was detected, primers could be designed for future use in PCR for this gene.

4. In this study, we had only investigated some of the antimicrobial resistance mechanisms of our enterococcal strains. Further work could be carried out to determine the possibility that isolates producing AAC6'-APH2" could produce one or more enzymes and the aminoglycoside resistance mechanisms of the four strains that gave no amplification products with specific primers for the gene coding for the bi-functional AME (AAC6'-APH2"). Primers specific for genes coding for other AMEs could be used. If subsequent PCRs yielded no amplification products, the resistance genes could be cloned and characterized. Alternatively, other mechanisms of resistance such as membrane permeability could be investigated.

5. None of our ampicillin-resistant enterococci produced detectable β-lactamases. Penicillin-binding proteins (PBPs) of these isolates could be characterized to determine if the resistance was due to presence of a PBP5 or other new altered PBPs.

6. During the incidence of VRE, we screened faecal samples of all patients in that particular ward where VRE was isolated to detect the present of VRE in other patients. VREs were isolated from other patients in the case of Van A VRE. Thus, we could apply the PFGE method on these isolates and investigate retrospectively the source and spread of the VRE.

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